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Review

Separation methods for methotrexate, its structural analogues and metabolites

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Abstract

Methotrexate (MTX) is the prototype folate antagonist cytotoxic drug, employed in the therapy of solid tumors and leukaemias, and recently also as an immunosuppressive agent in organ transplantation, in the treatment of some autoimmune diseases and in the therapy of severe asthma. MTX is one of the very few antineoplastic drugs the therapeutic concentration monitoring of which is currently employed in clinical practice and can be routinely measured in biological samples by a number of different analytical techniques, among which are immunoenzymatic and chromatographic methods. Each technique has of course its own advantages in terms of sensitivity, specificity, speed, cost and level of expertise required. Along with therapeutic drug concentration monitoring and clinical pharmacology, fundamental research into the mechanism of action of antifolate drugs is still a field which requires the measurement of MTX, of its new analogues and of their metabolites in biological samples. This review summarizes the instrumental conditions and the performance of several published chromatographic methods employed to measure MTX, its metabolites and some analogues in clinical and biological research. More than 70 papers describing chromatographic assays for MTX and its metabolites have been published in the literature between 1975 and 2000. A wide array of experimental conditions for sample preparation, analyte separation and detection have been employed. According to their chemical properties, MTX, its metabolites and analogue drugs present in several biological samples (plasma, serum, saliva, urine, cerebrospinal fluid, tissue specimens) can be extracted, separated and detected under a variety of chromatographic conditions, i.e. on different stationary phases, under a wide choice of mobile phase conditions (acidic or neutral, employing ion-pair or micellar chromatography), followed by several detection techniques (UV–Vis spectrophotometry, pre- or post-column oxidation and fluorimetry, electrochemistry, mass spectrometry). Optimized methods allow simultaneous measurement within a few minutes of the plasma levels of MTX and its main metabolites at concentrations in the low-n*M* range. One special field which needs sensitive, fast and inexpensive methods for the detection and measurement of MTX is the monitoring of contamination in workplace environments, such as pharmaceutical industries and oncological hospital pharmacies, and in sewage waters. The measurement of the intracellular γ-oligo-glutamate metabolites of biological folates, of MTX and of some analogue drugs is of great importance in basic pharmacological research. The existence of empirical quantitative relationships between the retention of individual oligomers under different chromatographic conditions and the number of added glutamic acid units allows identification of the metabolites even when authentic standards are not available.

Keywords: Reviews; Methotrexate; Folates

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Contents

1. Introduction

Methotrexate (MTX; amethopterin) is a cytotoxic drug which has been in use since the 1940s in the therapy of solid tumors and leukaemias, and more recently as an immunosuppressive agent in organ transplantation, in the treatment of autoimmune diseases, such as psoriasis and rheumatism, and in the therapy of severe asthma.

In the following, a short presentation will be made

of some chemical properties of methotrexate and of the naturally occurring related compounds (folates) and of the pharmacological mechanisms underlying the therapeutic activity of MTX [1], and of some newer analogs recently introduced into clinical use, as this information is relevant to their determination in biological matrices. This information also aims to highlight several facets of the continuing research interest into this molecule, which prompts for the availability of measurement techniques tailored to the various applications.

The aims of the analytical methods for the measurement of MTX are disparate, ranging from quality control and industrial hygiene purposes in the pharmaceutical industry, to animal and clinical pharmacokinetics, to therapeutic drug monitoring and to basic research in the chemical and biochemical fields.

1.1. Chemical properties of methotrexate, natural folates and antifolate drugs

The structures of methotrexate and of its main metabolites are collected in Fig. 1, and those of some folic acids in Fig. 2. The molecule of MTX (structure 1a) is made up of a heterocyclic portion (a 2,4-diamino-substituted pterine ring) linked to a *p*-aminobenzoyl portion, which is in turn amide bonded to a glutamic acid unit. The molecule is thus a polyelectrolyte carrying two carboxyl groups — with dissociation constants (pK_a) of 3.36 (α-carboxyl) and 4.70 (γ-carboxyl) — and a number of potentially protonated nitrogen functions, the most basic of which is presumably the guanidinic *N*-1 on the pterine ring (pK_a 5.71). Its water solubility is pH-dependent, ranging from 0.9 m*M* at pH 5 to 20 m*M* at pH 7. As apparent, the main structural variations of MTX with respect to the structure of the naturally occurring folic acid (structure 2a) are substitution of the hydroxyl group on C^2 with an amino function and methylation of N^{10}.

A number of structural analogues of MTX are investigated as new candidate antifolate drugs [2], and one (raltitraxed) is currently licensed for therapeutical use. These compounds can be classified into two broad categories: ''classical'' antifolates, prototyped by MTX, which retain an aryl-glutamate or a chemically equivalent group in their structure and ''non-classical'' antifolates, which lack the hydrophilic portion of the molecule. The structures of some of the most important drug candidates are reported in Fig. 3 for ''classical'' antifolates and in Fig. 4 for ''non-classical'' antifolates; their chemical and pharmacological characteristics, as relevant to

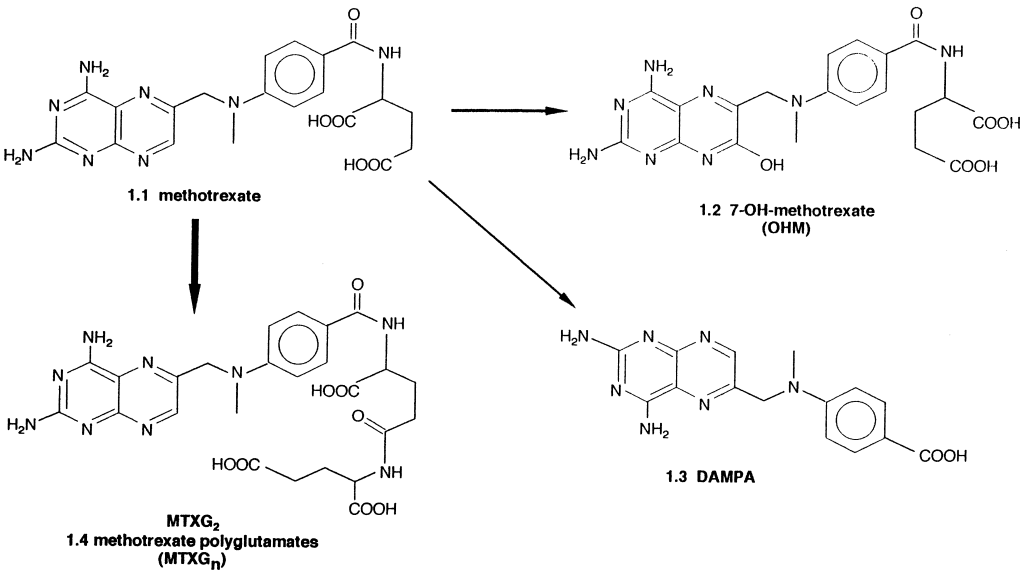


Fig. 1. Structures of methotrexate and its major metabolites.

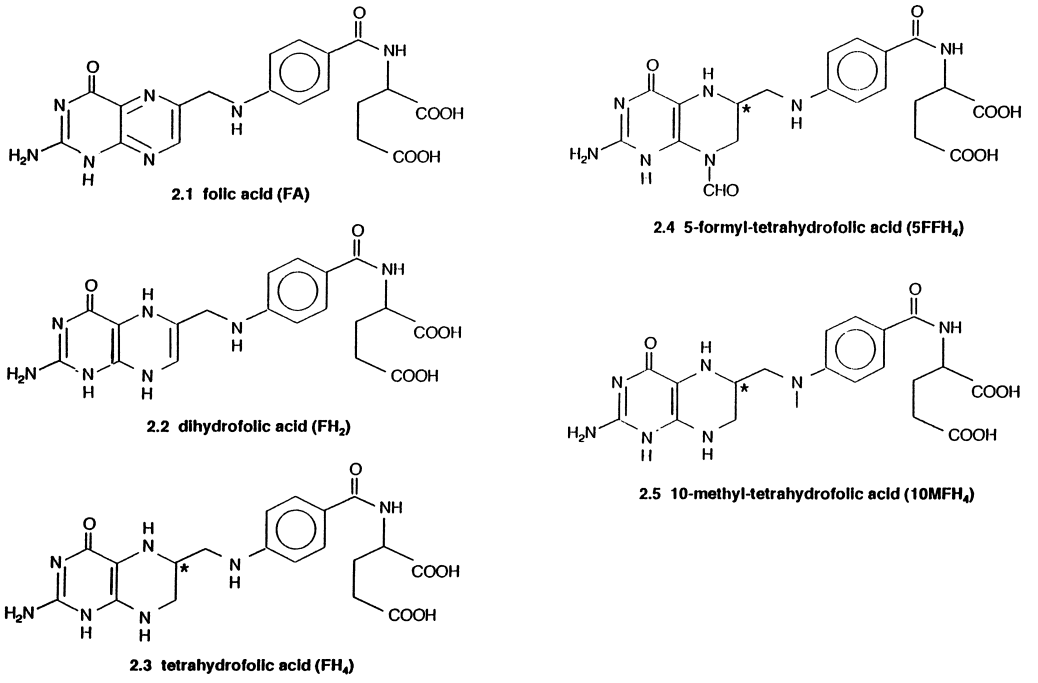


Fig. 2. Structures of some folic acids.

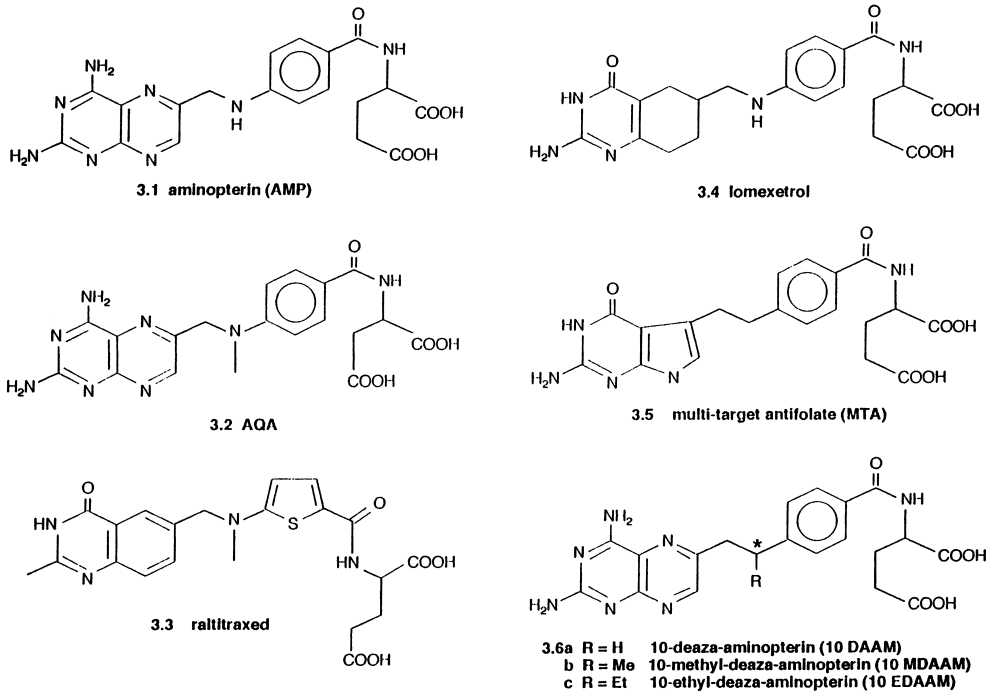


Fig. 3. Structures of some ‘‘classical’’ antitumor antifolate drugs related to methotrexate.

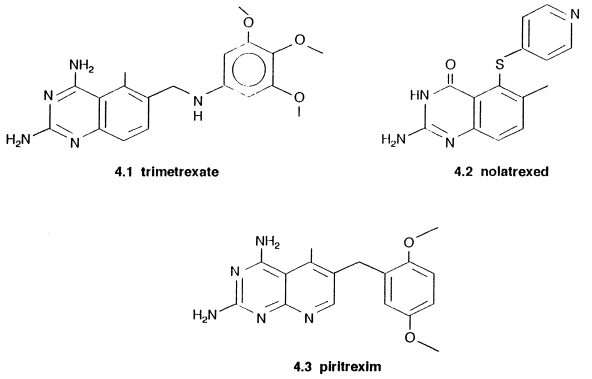


Fig. 4. Structures of some ''non-classical'' antitumor antifolate drugs.

outline the assays for their measurement in biological samples, will be summarized in Section 3.

1.2. Pharmacology of methotrexate

MTX is therapeutically administered both by the oral and the parenteral route. Low doses of MTX (<25 mg/m^2 of body surface) are readily absorbed from the gastrointestinal tract, while higher doses (up to 3 g/m^2) are not completely absorbed by this way and are therefore administered by intravenous infusion. MTX is readily distributed to the body tissues and its elimination from the bloodstream follows tri-phasic pharmacokinetics, with a $t_{1/2}$ of the adsorption phase within tens of minutes, of the distribution phase of approx. 2 h and of the elimination phase of approx. 8 h.

Due to its high polarity (at the neutral pH of biological fluids it is present mainly in the doubly anionic species), MTX can cross the cell membrane only with difficulty, and is therefore transported into the cells by a specific, high-affinity carrier system. Inside the cell, MTX follows the same biochemical pathways as folic acids, i.e. polyglutamylation by specific enzymes (folate-γ-glutamyl-transferases), which serially add one to four (or even more) residues of glutamic acid, linked by an iso-peptidic bond to the γ-carboxyl (structure 1d). The main pharmacological target of the action of MTX (and of a small number of similar drugs) is the competitive inhibition of dihydrofolate-reductase (DHFR), an intracellular enzyme which reduces folic acids to tetrahydro-folate cofactors, which are in turn key intermediates in several important biochemical pathways, among which are the de-novo biosynthesis of purines and of tymidilate. Lack of reduced folates, of purines and of thymine in actively proliferating cells, such as those of the tumors, leads to a blockage of DNA and RNA synthesis, and eventually to cell death.

The affinity of folate and MTX to some folate-requiring enzymes in the biosynthetic pathways towards purines and tymidilate has been found to increase as the number of γ-glutamyl residues increases. Moreover, the increased polarity of the poly-glutamylated species of MTX (MTX-G_n; Fig. 1.4) hampers their efflux from cells, in order that their presence in tissues such as the kidney and the liver can be detected several weeks later or even months after administration.

Since MTX inhibits a key cellular function, it is a very cytotoxic compound, especially towards actively replicating cells. Its cytotoxic activity is exerted not only towards neoplastic cells, but also targets other tissues, thus determining bone-marrow depression, inflammation of the upper gastrointestinal tract (mucositis) and severe gastrointestinal disturbance, especially in patients treated at the higher doses now employed to improve its efficacy on some tumors.

To overcome the dose-limiting toxicity of MTX, and to allow the use of protocols with very high doses of MTX (0.25–7 g/m^2 per week) for the treatment of resistant cell lines or to achieve cytotoxic drug concentrations across the blood–brain barrier, a strategy named ''folinic acid rescue therapy'' has been devised. Although the details of the involved biochemical mechanism is still debated, intravenous administration of leucovorin (chemically, a 1:1 racemic mixture of the [6*R*]- and [6*S*]- isomers of folinic acid, the latter stereoisomer being also known as citrovorum factor) at a scheduled time after the infusion of high-dose MTX therapy is beneficial to healthy cells and protects them from the cytotoxic action of MTX.

Low doses of MTX are poorly metabolized in the liver, while at higher doses two main metabolites are produced in humans and in experimental animals: 7-hydroxy-MTX (OHM; Fig. 1.2) and 4-amino-4-deoxy-N^{10}-methylpteroic acid (DAMPA; Fig. 1.3). OHM, produced by hepatic aldehyde oxidase [3,4],

is even less soluble than MTX in the acidic environment of the proximal renal tubule and can precipitate, thus causing renal damage. To circumvent this risk, it is advisable to treat patients with sodium bicarbonate to alkalinize the urine. DAMPA is a decomposition or by-product present at levels below 1% in MTX pharmaceutical formulations [5,6], and, since it is measured in blood and urine of treated patients at much higher concentrations than justified by its presence in MTX formulations, studies have demonstrated that it is a metabolite of MTX produced by the intestinal flora [7]. Since the solubility of DAMPA is one-tenth of that of MTX, this metabolite may also cause renal damage.

MTX is one of the very few antineoplastic drugs for which therapeutic drug concentration monitoring is currently employed in clinical practice, since a careful monitoring of plasma concentrations of MTX is required, especially in high-dose MTX protocols including folinic acid rescue. MTX can be measured in biological samples by a number of different analytical techniques: bioassays, immunoenzymatic methods (FPIA), chromatography, each one of which having its own advantages in terms of sensitivity, specificity, speed, cost and level of required expertise to be performed. Moreover, the search for monitoring of therapeutic drug levels in easy to collect biological fluids, such as saliva, which are an alternative to the invasive blood venipuncture has triggered interest. Furthermore, since folinic acids are often administered concurrently to MTX, therefore analytical methods for the simultaneous determination of folates as well as MTX and its metabolites can be of applicative value.

Another recent field of interest which requires low-level measurement of MTX in environmental and biological samples is the occupational hygiene monitoring of the contamination level of workplaces, such as production facilities in the pharmaceutical industry and hospital pharmacies and administration rooms, as well as the biological monitoring of exposed workers [8].

1.3. Aim of the review

There is a lack of recent reviews on the subject of chromatographic determination of methotrexate and of its analogues, although more than 40 analytical papers have been published on this subject after publication of a review in 1985 [9] and of several others before, on the measurement of MTX [10,11] and of the natural folates [12,13]. This review summarizes the instrumental conditions and the performance of more than 70 liquid chromatographic methods (published between 1975 and 2000) employed to measure MTX and its metabolites in biological samples and of a few assays employed for the quality control of MTX drug formulations and for the determination of MTX contamination in environmental samples.

Other papers considered report assays for newer MTX-analogue drugs (the ''classical'' antifolates raltitraxed, lomexetrol, multi-targeted antifolate and the ''non-classical'' trimetrexate, nolatrexed and piritrexim). Another related topic which has been reviewed is the determination of the γ-polyglutamate oligomers of natural folates, of MTX (MTX-G_n) and of the ''classical'' antifolates, which are the main intracellular metabolites.

A few reports of the use of capillary electrophoretic methods have also been reported, as cited in Ref. [9], but a discussion of their merits has been purposely omitted from this report, due to the fact that the required instrumentation, although commercially available at a cost comparable to that of conventional HPLC equipment, is still uncommon in analytical pharmacology laboratories. From a merely speculative point of view, one of the merits of the technique is the almost negligible cost of the consumables (columns and solvents) and the use of aqueous buffers for separation makes the technique attractive in clinical chemistry environments, once the problems related to sensitivity, sample turnaround time and assay automation have been solved.

One of the main messages this review aims to share with the reader is to highlight how an ingenious choice and the assembly of the individual steps of sample preparation, conditions for analyte separation and detection are able to tailor the global assay to the needs of the individual user, to the available instrumentation and to the professional level of laboratory technicians.

In fact, it is often overlooked that the analytical needs are not always pushed to extremes of sensitivity, specificity and speed (which are unavoidably paid for by an increase in the analyst's ''fourth s'', $),

Table 1
Analytical conditions of 19 chromatographic assays for methotrexate in biological matrices and other samples

Sample	Prep.	IS	Column type	Pack (μm)	L (mm)	D (mm)	I/G	Buffer composition	pH	%	Org. modif.	%	Flow (ml/min)	Temp. (°C)	Det.	Run time (min)	Analyte	Ret. time (min)	Recovery (%)	LoD/LoQ	Calibration min micromolar	Range max.	Accuracy (% of target)	Precision (CV%)	Ref.
S, QC	dir	n	Vydac anion-exchange	nr	500	2.1	AE	0.3 *M* $NaClO_4$ 0.15 *M* NaH_2PO_4 0.15 *M* Na_2HPO_4	nr	99.5	ACN	1.5	0.6	RT	UV (254)	15	MTX	7.5	nr	2.2E+00	nr	nr	1	nr	[14]
P	on-line	n	Partisil SAX	10	250	4.6	AE	0.05 *M* PB 0.001 *M* NaCl	4.9	80	MeOH	20	2.5	RT	UV (306)	15	MTX	5.5	70	2.0E−02	2.0E−02	5.0E+01	nr	2–5	[15]
QC	dir	n	m-Bondapack C_{18}	nr	300	4.6	I	0.005 *M* NH_4OAc	5.0	85	MeOH	15	nr	RT	UV (254)	50	MTX	12	nr	nr	nr	nr	nr	nr	[16]
P	on-line	AQA	Inertsil ODS-2	5	150	4.6	I	0.02 *M* PB	7.0	82	ACN	18	1	RT	UV (305)	40	MTX	24.5	nr	1.1E−02	1.1E−02	2.2E+00	−1.2–0.5	1.1–12%	[17]
P	prec-TCA	n	mu-Bondapak C_{18}	nr	300	4	I	0.1 *M* Tris–P	6.7	80	MeCH	20	1	RT	UV (305)	10	MTX	8	40	8.9E−01	nr	nr	nr	nr	[18]
P	prec-TCA ox-$KMnO_4$	CHF	mu-Bondapak C_{18}	nr	300	4	I	0.1 *M* Tris–P	6.7	100			1	RT	F (275/410)	15	MTX OHF	9 4.5	40	2.2E−02	4.4E−02	2.2E+01	±10	nr	[18]
S	on-line	n	Lichrosphere RP-18	5	125	4	I	0.05 *M* PB 0.005 *M* TBA	7.4	82	ACN	18	1	RT	UV (307)	15	MTX	10	nr	2.2E−02	nr	nr	nr	nr	[19]
S, sal, CSF	prec TCA-EtOH	pAAP	Novapak C_{18}	4	100	8	I	0.01 *M* Tris 0.1 *M* HNa_2PO_4	5.8	84	MeOH ACN	11 5	2.3	RT	UV (313)	10	MTX	5.3	98–71	2.2E−02	2.2E−02	2.2E+03	nr	<5	[20]
P	prec-ACN cooling	pAAP	Radialpak C_{18}	5	nr	nr	I	0.1 *M* Na_2HPO_4 0.01 *M* Tris-P	5.8	82	MeOH ACN	13 5	2.3	RT	UV (313)	11	MTX	5	96	4.4E−02	5.0E−02	1.0E+01	−8.6+5.6%	3.8	[21]
S, U	dir	n	Lichrosphere 100 RP-18	5	125	4	I	0.1 *M* SDS 0.05 *M* NaH_2PO_4 0.05 *M* NaH_2PO_4	5.7	100			1	RT	UV-DAD	7	MTX	2.1	100	1.8E−03	1.0E−01	1.0E+02	nr	4.2	[22]
S, P	prec-H_2SO_4 extr EA-iP	pAAP	ZORBAX SP-150 C_8	nr	250	4.6	I	0.1 *M* PB	6.9	80	MeOH	20	1	RT	EC	20	MTX	5.5	38	nr	2.2E+01	4.4E+04	nr	3	[23]
S, P	prec-TCA	teof	Brownlee RP18	10	250	4.6	I	0.05 *M* AcB	5.5	87	MeOH ACN	6.5 6.5	1.5	RT	EC	20	MTX	13	96	1.1E+00	nr	nr	nr	7	[24]
S	SPE	teof	Brownlee RP18	10	250	4.6	I	0.05 *M* AcB	5.5	87	MeOH ACN	6.5 6.5	1.5	RT	EC	20	MTX	13	94	4.4E−02	nr	nr	nr	9	[24]
S	SPE	teof	Brownlee RP18	3	100	4.6	I	0.05 *M* AcB	5.5	85	MeOH ACN	8 7	0.8	RT	EC	20	MTX	13	94	2.2E−03	nr	nr	nr	9	[24]
P	SPE-IE	teof	Brownlee RP18	10	250	4.6	I	0.05 *M* AcB	5.5	87	MeOH ACN	6.5 6.5	1.5	RT	EC	20	MTX	13	80	4.4E−03	1.1E−02	4.4E−00	nr	4.6	[24]
S	prec-PCA ox $KMnO_4$	n	mu-Bondasphere 100A	nr	150	3.9	I	0.005 *M* TBA–HSO_4	nr	95	MeOH	5	1	50	F (280/459)	9	MTX	3.8	nr	nr	2.2E−02	2.2E+00	nr	nr	[25]
P	on-line	nr	ODS-TM	nr	nr	nr	I	0.05 *M* PB	6.6	90	ACN	10	nr	RT	pcox-F (367/463)	15	MTX	nr	94–98	nr	1.1E−01	1.1E−00	nr	<4	[26]
S	prec-ACT extr EE-nB	AMP	Custom glass 1G-ODS	58	250	4.6	I	0.01 *M* AcB	3.8	88	MeOH	12	1.5	RT	UV (305)	25	MTX AMP	14 7.5	nr nr	1.5E−01 nr	nr nr	nr nr	nr	nr	[27]
Env	dir SPE	AMP	Supelcosil RP-18	3	43	4.6	I	0.01 *M* NH_4OAc	6.0	96	ACN	4	2	40	UV (310)	8	MTX AMP	6.2 1.6	93 nr	1.0E+02 nr	1.4E+02 nr	2.2E+03	−5.9+7.3%	2–15	[28]
P	dir	n	Hypersil MOS	5	100	2.1	G	H_2O/H_3PO_4/TEA 99.85:1:0.5	2.2		ACN	0–60 in 5 min	1	40	UV (303)	10	MTX	2.98	100	1.0E−02	5.0E−02	2.0E+00	nr	0.9–4.3	[29]
U	SPE+ on-line	n	Lichrosphere RP-18	10	250	4	G	314 mM NH_4OFor	2.7		ACN	1–25	1	25	UV (310)	40	MTX	35	97–99	nr	8.9E−03	2.2E+03	nr	2.2–4.1	[30]

Abbreviations: AcB, acetate buffer (acetic acid–Na acetate); AE, anion-exchange; CHF, chloroform; CSF, cerebro-spinal fluid; dir, direct sample injection; EA-iP, ethyl acetate–iso-propanol mixture; EC, electrochemical detection; EE-nB, diethyl ether–*n*-butanol mixture; env, environmental sample; IE, ion-exchange; I/G, isocratic or gradient; IS, internal standard; NH_4OAc, ammonium acetate; nr, not reported; OHF, hydroxy-folic acid; P, plasma; pAAP, *p*-amino-acetophenone; PB, phosphate buffer; PCA, perchloric acid; pcox-F, post-column photooxidation–fluorimetric detection; prec, precipitation; RT, room temperature; S, serum; sal, saliva; TCA, trichloroacetic acid; teof, theophylline; U, urine; UV(nm), ultraviolet detection (wavelength); 3BM, 3-bromo-MTX; 8CT, 8-chlorotheophylline.

Table 2
Analytical conditions for 38 chromatographic assays for the measurement of methotrexate and 7-hydroxy-methotrexate in biological matrices and other samples

Sample	Prep.	IS	Column type	Pack (μm)	L (mm)	D (mm)	I/G	Buffer composition	pH	%	Org. modif.	%	Flow (ml/min)	Temp. (°C)	Det.	Run time (min)	Analyte	Ret. time (min)	Recovery (%)	LoD/LoQ	Calibration min micromolar	Range max.	Accuracy (% of target)	Precision (CV%)	Ref.
S	Prec-PCA extr EA-iP	AQA	Partisil SAX	10	250	4.6	AE	0.025 *M* PB	7	100			1.2	RT	UV(315)	15	MTX	7.3	46	1.0E−01	2.0E−01	2.0E+01	nr	3	[31]
																	OHM	8.9	26	nr	1.0E+00	3.0E+01	nr	nr	
CQ, U	Dir	n	mu-Bondapak C_{18}	nr	300	4	I	0.005 *M* TBAP (PIC A)	7.5	80	MeOH	20	0.45	RT	UV(254)	150	MTX	32	nr	nr	nr	nr	nr	nr	[32]
																	OHM	47	nr	nr	nr	nr	nr	nr	
S, CSF	Prec-ACN extr EE-nB	n	mu-Bondapak C_{18}	nr	nr	nr	I	0.15 *M* AcB	4.6	89	ACN	11	2	RT	UV(303)	10	MTX	6	70	1.1E−02	2.0E−02	6.0E+03	6	3.5–7.7	[33]
																	OHM	8.4	nr	nr	nr	nr	nr	nr	
P	On-line	n	Partisil SAX	10	250	4.6	AE	0.05 *M* PB	4.9	80	MeOH	20	nr	RT	UV(306)	10	MTX	4.5	65.1	nr	nr	nr	nr	nr	[34]
																	OHM	6.5	70.2	nr	nr	nr	nr	nr	
S	SPE	AQA	mu-Bondapak C_{18}	nr	300	4	I	0.2 *M* AcB 0.03 *M* EDTA	5.5	85	MeOH ACN	8.4 6.6	1	RT	UV(313)	15	MTX	9	90	nr	2.2E−01	2.2E+00	nr	nr	[35]
																	OHM	10.5	nr	nr	nr	nr	nr	nr	
																	AQA	6	nr	nr	nr	nr	–	–	
P	Dir	n	mu-Bondapak C_{18}	nr	300	4	I	5 m*M* Bu_4NPO_4	7.5	64	MeOH	36	1	RT	UV(315)	20	MTX	11	nr	nr	1.0E+00	1.0E+02	nr	nr	[36]
																	OHM	13	nr	nr	1.0E+00	1.0E+02	nr	nr	
S	Prec-PCA extr EA-iP	pAAP	RP-8	10.7	250	4.1	I	0.1 *M* PB	6.8	85	MeOH	15	1.5	RT	UV(313)	12	MTX	6.5	46	1.1E−01	2.2E−01	1.8E+01	nr	5–10	[37]
																	OHM	7.5	26	nr	2.2E−01	1.8E+01	nr	nr	
S	Prec-TCA	AQA	custom Hypersil ODS	5	120	4	I	0.1 *M* Tris 0.1 *M* NaH_2PO_4	6.7	80	MeOH	20	1	RT	UV(305)	20	MTX	11.5	82.2	2.2E−01	1.1E+00	4.4E+00	nr	7.3–10.3	[38]
																	OHM	14.5	98.2	4.2E−01	1.1E+00	4.4E+00	nr	8.6–12.6	
S	SPE	AQA	mu-Bondapak C_{18}	nr	300	4	I	0.2 *M* AcB	5.5	85.3	MeOH ACN	8.4 6.3	1.5	RT	UV(313)	15	MTX	9	90	nr	2.2E−01	2.2E+02	nr	nr	[39]
																	OHM	12	70	nr	2.2E−01	2.2E+02	nr	nr	
S	SPE	8CT	Radial-PAK mu-Bondapak C_{18}	nr	nr	5	I	0.01 *M* PB	4.5	85	ACN	15	0.8	RT	UV(305)	10	MTX	7.5	82	6.0E−02	nr	nr	nr	8–15	[40]
																	OHM	5.5	72	1.2E−01	nr	nr	nr	7.6–8.9	
S	SPE	n	Radialpak C_{18}	10	100	5	I	0.15 *M* $NH_4H_2PO_4$	4.85	89	ACN	11	1	RT	UV(313)	10	MTX	5.8	70	2.2E−02	1.1E−01	1.1E+01	nr	6.4	[41]
																	OHM	7.9	nr	nr	nr	nr	nr	7.3	
P	SPE	n	nr	nr	nr	nr	I	PB	3	77	MeOH ACN	15 8	6	RT	nr	10	MTX	nr	nr	1.4E−02	nr	nr	7.8		[42]
																	OHM	nr	nr	nr	nr	nr			
P	Prec-ACN	n	mu-Bondapak C_{18}	10	300	4	I	0.07 *M* PB	7	95	ACN	5	2.5	25	UV(313)	25	MTX	16.3	74	2.0E−01	nr	nr	nr	8	[43]
																	OHM	19.8	81	3.3E+00	nr	nr	nr	7	
P	Prec-PCA	n	Supelcosil LC-18	5	250	4.6	I	0.1 *M* Tris–NaH_2PO_4	6.7	75	MeOH	25	1	RT	UV(370)	12	MTX	6.9		3.0E−02	1.0E−01	1.0E+01	nr	7.2	[44]
																	OHM	8.2		3.0E−02	1.0E−01	1.0E+01	nr	9.1	
S, U, eritr	Pre-ACT extr EE-nB	AMP	custom ODS-Hypersil	5	100	4.5	I	0.05 *M* NaH_2PO_4	8	96	THF	4	0.6	RT	UV(305)	5	MTX	4	63	1.1E−01	nr	2.2E+01	nr	5.6	[45]
																	AMP	2.5		nr	nr	nr			
P	Prec TCA-HCl	SFZ	Hibas Lichrosorb RP18	5	250	4	I	0.15 *M* $NH_4H_2PO_4$	4.85	87.4	ACN	12.6	1.5	RT	UV(313)	35	MTX	5	64	8.9E−02	8.9E−02	1.1E+01	nr	13	[46]
																	OHM	8	71	1.8E−01	1.8E−01	1.1E+01	nr	10	
P	SPE	8CT	Waters RCM 100 C_{18}	nr	nr	nr	I	0.01 *M* KH_2PO_4	3.9	87.5	ACN	12.5	2.5	RT	UV(313)	20	MTX	8	40–48	1.5E−02	nr	nr	8.5	8.5	[47]
																	OHM	5	nr	1.5E−02	nr	nr	7.1	8.5	

Table 2. Continued

Sample	Prep.	IS	Column type	Pack (μm)	L (mm)	D (mm)	I/G	Buffer composition	pH	%	Org. modif.	%	Flow (ml/min)	Temp. (°C)	Det.	Run time (min)	Analyte	Ret. time (min)	Recovery (%)	LoD/LoQ	Calibration min micromolar	Range max.	Accuracy (% of target)	Precision (CV%)	Ref.
S, CSF	SPE	n	Separon SIX C_{18}	5	150	3.2	I	0.01 *M* PB	6.8	80	MeOH	20	0.4	RT	UV(303)	15	MTX	10.3	93	5.0E−02	1.1E+00	3.5E+01	nr	nr	[48]
																	OHM	13.4	nr	nr	nr	nr	nr	nr	
S	Prec-TCA	AMP	Ultrasphere ODS RP-18	5	250	4.6	I	0.05 *M* NaH_2PO_4	4.85	95	THF	5	0.6	RT	UV(313)	25	MTX	15	>70	nr	nr	nr			[49]
																	OHM	16.5		nr	nr	nr			
																	AMP	12.5		nr	nr	nr			
S	Prec-ACT Extr EE-nB	AMP	Ultrasphere ODS RP-18	5	250	4.6	I	0.05 *M* NaH_2PO_4	4.85	95	THF	5	0.6	RT	UV(313)	25	MTX	15	>90	4.0E−06	2.2E−02	2.2E+01	4.2	2.6–7	[49]
																	OHM	16.5	nr	nr	nr	nr	nr	nr	
																	AMP	12.5		nr	nr	nr		2.2–4	
P, U	SPE	n	Spherisorb S3-ODS2	nr	100	4.6	I	0.1 *M* Na-phosphate+ 0.2% H_2O_2	6.5	94	DMF	6	1	RT	pcox-F (350/435)	15	MTX	6	88–103	2.0E−04	nr	1.0E−01	nr	5	[50]
																	OHM	10	88–103	1.0E−03	nr	4.6E−01	nr	5	
S	SPE	n	Intersil ODS-2	nr	nr	nr	G	PB	3		ACN		nr	RT	UV(303)	nr	MTX	nr	nr	2.2E−03	5.6E−03	2.2E−01	nr	4.4	[51]
																	OHM	nr	nr	2.2E−03	5.6E−03	2.2E−01	nr	4.6	
P	Prec-ACN Extr CHF	n	Lichrocart	5	250	4	I	0.025 *M* AcB	3.9	88	ACN	12	1.5	RT	UV(307)	15	MTX	6	88.5	8.9E−02	4.4E−01	3.7E+01	nr	5.3	[52]
																	OHM	10	nr	nr	nr	nr	nr	nr	
P	SPE	n	Novapak C_{18}	5	150	3.9	I	0.014 *M* PB 0.5% H_2O_2	6.5	92.7	ACN DMF	3.3 4.0	1	RT	pcox-F (350/465)	10	MTX	4	97–101	1.1E−04	2.2E−04	2.2E+00	nr	7.1	[53]
																	OHM	5.5	76–86	4.4E−03	1.4E−02	8.9E−01	nr	5.5	
P	SPE	n	C_{18} Perkin-Elmer	3	80	4.6	I	0.01 *M* PB+ 0.2% H_2O_2	6.5	94	ACN	6	1	RT	pcox-F (350/435)	30	MTX	nr	nr	1.0E−03	1.0E−03	5.0E−01	nr	6.1–10.5	[54]
																	OHM	nr	nr	nr	nr	nr	nr	<9.4	
S	SPE	8CT	Beckman Ultrasphere ODS	5	150	4.6	I	0.01 *M* KH_2PO_4 0.02 *M* TMAC	2.5	90	ACN	10	1	RT	UV(313)	30	MTX	13.3	88–91	3.0E−02	3.0E−02	1.0E+00	−1.4±12.6	2.7–14.4	[55]
																	OHM	20.7	nr	nr	nr	nr	nr	nr	
P	On-line	n	Lichrosphere RP-18	5	125	4	I	0.05 *M* PB 0.005 *M* TBA	7.4	85	ACN	15	1	RT	pcox-F (350/417)	30	MTX	15	100	8.0E−04	1.0E-02	5.0E+00	−6±6	<4.4	[56]
																	OHM	18	nr	nr	nr	nr	nr	nr	
U	SPE	n	Inertsil ODS-2	5	150	4.6	I	0.05 *M* PB	5.3	90.5	ACN	9.5	1.1	RT	UV(303)	20	MTX	6.8	80	1.1E−02	1.1E−02	2.2E+00	−3.6–2.6	<8.4	[57]
																	OHM	10.2	70	1.1E−02	1.1E−02	2.2E+00	−2.8–2.6	<7.8	
P	Prec-ACN extr CHF	ID	Kromosil C_8	5	50	1	I	0.1% HCOOH	2	80	ACN	20	0.15	RT	MS2 MH±147	1.75	MTX	0.60	61	1.1E−04	2.2E−04	5.6E−01	2.7–10.2	3.2–7.4	[58]
																	OHM	0.70	46	2.2E−04	5.6E−04	2.2E−01	0.2–3.6	3.8–7.6	
P	Prec-ACT extr EE-nB prec MeOH	n	Inertsil ODS-80A	5	150	4.6	I	$HClO_4$ in H_2O	2	95	THF	5	1	60	UV(313)	30	MTX	9.3	nr	2.2E−02	2.2E−02	2.2E+01	−4.5±10.6	5.4–11	[59]
																	OHM	16.5	nr	nr	nr	nr	nr	nr	
P	SPE	AMP	PS–DVB (PRP-1)	5	150	4	I	0.1 *M* PB+ 0.2% H_2O_2	6.5	94	DMF	6	1	RT	pcox-F (350/435)	20	MTX	10	86–111	2.2E−03	5.6E−03	1.1E−01	0–10.5	1.8–8.2	[60]
																	OHM	13	91–102	5.6E−03	5.6E−03	1.1E−01	0–10.5	4–6.8	
																	AMP	5.5		nr	nr	nr			
S, CSF	Prec PCA	n	Supelcosil C_{18}	3	150	4.6	I	0.1 *M* Tris–NaH_2PO_4	6.7	82	MeOH	18	1	40	UV(370)	10	MTX	6.8	70	2.0E−02	1.0E−01	1.0E+03	nr	<4	[61]
																	OHM	7.8	70	2.0E−02	2.0E−02	1.8E+02	nr	<8	
U	SPE	OHM	Discovery C_{18}	5	150	4.6	I	0.02 *M* NH_4OAc	4	70	MeOH	30	1	RT	MS2 MH±147	8	MTX	2.51	100	4.4E−04	1.1E−03	3.6E−02	100–112	6.7–17	[62]
																	OHM	3.81		nr	nr	nr			
env	SPE	OHM	Discovery C_{18}	5	150	4.6	I	0.02 *M* NH_4OAc	4	70	MeOH	30	1	RT	MS2 MH±147	8	MTX	2.45	nr	1.1E−02	1.1E−02	3.6E−01	97–115	5.9–16	[63]
																	OHM	3.81		nr	nr	nr			

Abbreviations: AcB, acetate buffer (acetic acid–Na acetate); AE, anion-exchange; CHF, chloroform; CSF, cerebro-spinal fluid; dir, direct sample injection; EA-iP, ethyl acetate–iso-propanol mixture; EC, electrochemical detection; EE-nB, diethyl ether–*n*-butanol mixture; env, environmental sample; IE, ion-exchange; I/G, isocratic or gradient; IS, internal standard; NH_4OAc, ammonium acetate; nr, not reported; OHF, hydroxy-folic acid; P, plasma; pAAP, *p*-amino-acetophenone; PB, phosphate buffer; PCA, perchloric acid; pcox-F, post-column photooxidation-fluorimetric detection; prec, precipitation; RT, room temperature; S, serum; sal, saliva; TCA, trichloroacetic acid; teof, theophylline; U, urine; UV(nm), ultraviolet detection (wavelength); 3BM, 3-bromo-MTX; 8CT, 8-chlorotheophylline.

Table 3
Analytical conditions for 15 chromatographic assays for the measurement of methotrexate, 7-hydroxy-methotrexate, diamino-pteroic acid and some reduced folates in biological matrices

Sample	Prep.	IS	Column type	Pack (μm)	L (mm)	D (mm)	I/G	Buffer composition	pH	%	Org. modif.	%	Flow (ml/min)	Temp. (°C)	Det.	Run time (min)	Analyte	Ret. time (min)	Recovery (%)	LoD/LoQ	Calibration min micromolar	Range max.	Ref.
P	Prec-PCA extr EA-iP	n	Partisil PXS 10/25 ODS	nr	250	5	CE	5 m*M* hexanesulfonic acid	3.8	70	MeOH	30	1.0	RT	UV(305)	30	MTX	11	nr	2.2E−01	nr	nr	[64]
																	OHM	9	nr	nr	nr	nr	
																	DAMPA	28	nr	nr	nr	nr	
P, U	Prec-PCA extr EA-iP	n	Partisil PXS 10/25 ODS	nr	250	5	CE	5 m*M* hexanesulfonic acid	3.8	70	MeOH	30	1.0	RT	UV(305)	30	MTX	2	nr	nr	4.4E+00	2.2E+02	[65]
																	OHM	3	nr	nr	4.4E+00	4.4E+00	
																	DAMPA	8	nr	nr	nr	nr	
S, U, sal	Prec-ACN extr EA-iAA	n	Partisil PXS 10/25 SCX	10	250	5	CE	0.02 *M* $(NH_4)_2HPO_4$	3.0	90	ACN	10	2.0	RT	UV(313)	12	MTX	7	70	3.3E−02	2.2E−01	2.2E+01	[66]
																	OHM	5	50	5.6E−02	2.2E−01	2.2E+01	
																	DAMPA	9	77	2.2E−02	2.2E−01	2.2E+01	
S, P, CSF	Prec-ACN extr EE-nB	n	mu-Bondapak Phenyl	nr	250	4	I	0.15 *M* NaOAc	4.6	89	ACN	11	2.0	23	UV(303)	12	MTX	6	70	1.5E−02	7.0E−02	1.0E+02	[67]
																	OHM	9	nr	nr	nr	nr	
																	DAMPA	18	nr	nr	nr	nr	
S, U, bile	SPE	3BM	Novapak C_{18}	4	150	4	I	0.05 *M* PB	2.7	88	ACN	12	1.5	20	UV(303)	16	MTX	5	93–97	2.2E−03	1.5E−02	4.8E+00	[68]
																	OHM	6	78–81	2.2E−03	1.7E−02	9.3E−01	
																	DAMPA	10	88–92	2.2E−03	1.6E−02	1.0E+00	
P, U, CSF	Dir	n	Spherisorb ODS	5	150	4	I	0.05 *M* citric acid 0.05 *M* NaH_2PO_4	3.2	80	MeOH THF	16 4	2.0	RT	UV(303)	8	MTX	3	90–112	1.0E−01	1.0E−01	1.0E+03	[69]
																	OHM	5	versus aq.	nr	nr	nr	
																	DAMPA	8	std. sol.	nr	nr	nr	
P, U, bm	Prec-TCA	AMP	Lichrosorb 5RP-18	nr	nr	nr	I	0.067 *M* AcONa 5% AcOH	2.6	88	ACN	12	0.8	RT	UV(313)	20	MTX	13	nr	2.0E−02	nr	nr	[70]
																	OHM	15	nr	nr	nr	nr	
																	DAMPA	18	nr	nr	nr	nr	
																	AMP	nr	nr	nr	nr	nr	
P	Prec TCA-AcOH extr EA	teof	Lichrosorb RP-18	7	250	5	I	0.05 *M* NH_4OAc	5.0	87	MeOH ACN	6.5 6.5	1.8	RT	UV(305)	36	MTX	9	96	2.2E−01	4.4E−01	1.1E+02	[71]
																	OHM	15	95	nr	nr	nr	
																	DAMPA	31	92	nr	nr	nr	
P, U, sal	SPE	n	C_{18} Perkin-Elmer	3	80	5	I	0.01 *M* PB+0.2% H_2O_2	6.5	94	ACN	6	1.0	RT	pcox-F (350/435)	30	MTX	11	86	nr	2.0E−04	5.0E−01	[72]
																	OHM	13	91	nr	2.0E−04	5.0E−01	
																	DAMPA	23	102	nr	2.0E−04	5.0E−01	
P, U, asc	Dir	n	Lachema Silasorb C_{18}	10	250	5	I	0.05 *M* PB+0.15% H_2O_2	6.2	90	ACN	10	1.0	45	pcox-F (360/417)	10	MTX	5	nr	2.0E−02	5.0E−02	1.0E+00	[73]
																	OHM	6	nr	3.0E−02	nr	nr	
																	DAMPA	9	nr	3.0E−02	nr	nr	

Table 3. Continued

Sample	Prep.	IS	Column type	Pack (μm)	L (mm)	D (mm)	I/G	Buffer composition	pH	%	Org. modif.	%	Flow (ml/min)	Temp. (°C)	Det.	Run time (min)	Analyte	Ret. time (min)	Recovery (%)	LoD/LoQ	Calibration min micromolar	Range max.	Ref.
S	Dir	n	Lichrosorb RP-18	10	250	5	I	500 ml 0.05 *M* P+ 35 ml ACN+28 ml DMF	6.2				1.0	45	pcox-F (370/417)	10	MTX	5	nr	8.9E−04	nr	1.7E−01	[74]
																	OHM	5		2.20E−03	nr	1.6E−01	
																	DAMPA	8		1.3E−03	nr	1.1E−01	
S, U, CSF	Prec TCA-EtOH	AMP	mu-Bondapak C_{18}	10	300	4	G	0.1 *M* ammonium formate	3.5		ACN		2.0	RT	UV(308)	40	MTX	12	nr	4.8E−02	nr	nr	[75]
																	OHM	22	nr	3.2E−01	nr	nr	
																	DAMPA	15	nr	1.8E−01	nr	nr	
																	AMP	9		nr	nr	nr	
S, U, bile	Dir	n	Partisil ODS-2	nr	nr	nr	I	0.1 *M* NaOAc	3.6	85	ACN	15	1.0	RT	UV(254)	nr	MTX	9	nr	nr	nr	nr	[76]
																	MTHHF	15	nr	nr	nr	nr	
S, CSF	SPE	n	Supelcosil LC-18-DB	3	150	5	G	(A) 5 m*M* KH_2PO_4–H_3PO_4 (B) ACN	2.3				0.9	RT	UV(310)	33	MTX	23	100–103	2.2E−02	2.2E−02	1.1E+01	[77]
																	OHM	26	93–98	2.2E−02	2.2E−02	1.1E+01	
															F		5MFH4	6	94–95	4.4E−02	4.4E−02	1.1E+01	
															295/355		5FFH4	14	nr	2.2E−01	2.2E−01	1.1E+01	
U	SPE	n	mu-Bondapak C_{18}	nr	300	4	I	0.01 *M* AcB	5.5	75	MeOH	25	1.5	RT	UV(254)	30	MTX	6	77–90	nr	nr	nr	[78]
																	OHM	11	nr	nr	nr	nr	
																	DAMPA	23	78–89	nr	nr	nr	
																	LV	4	nr	nr	nr	nr	
																	MFH4	5	nr	nr	nr	nr	
P, CSF	SPE	n	Hypersil ODS Chrompak	5	200	3	G	(A) 10 m*M* NH_4-For (B) 75% 10 m*M* NH_4-For 25% ACN	3.5			15–95 B in 21 min	0.4	RT	UV(305)	33	MTX	22	80–81	7.0E−06	nr	5.0E+01	[79]
																	OHM	27	77	7.0E−06	nr	5.0E+01	
																	MFH4	15	91–94	8.0E−07	nr	5.0E+01	
																	LV	17	52–66	5.0E−07	nr	5.0E+01	
																	AMP	18	85	nr	nr	nr	
Sewage water	SPE	n	Nucleosil C_{18}	5	250	4	G	0.01 *M* KH_2PO_4–H_3PO_4	3.0	st: 97 end: 25	MeOH	st: 3 end: 75	1.0	RT	UV-DAD	35	MTX	10	nr	8.9E−01	nr	nr	[80]

Abbreviations: AcB, acetate buffer (acetic acid–Na acetate); AE, anion-exchange; CHF, chloroform; CSF, cerebro-spinal fluid; dir, direct sample injection; EA-iP, ethyl acetate–iso-propanol mixture; EC, electrochemical detection; EE-nB, diethyl ether–*n*-butanol mixture; env, environmental sample; IE, ion-exchange; I/G, isocratic or gradient; IS, internal standard; NH_4OAc, ammonium acetate; nr, not reported; OHF, hydroxy-folic acid; P, plasma; pAAP, *p*-amino-acetophenone; PB, phosphate buffer; PCA, perchloric acid; pcox-F, post-column photooxidation-fluorimetric detection; prec, precipitation; RT, room temperature; S, serum; sal, saliva; TCA, trichloroacetic acid; teof, theophylline; U, urine; UV(nm), ultraviolet detection (wavelength); 3BM, 3-bromo-MTX; 8CT, 8-chlorotheophylline.

since the sensitivity of the assay and often even the accuracy and precision of the results need only to be adequate to their end-use, which may be in the field of regulated experimental or clinical pharmacology, in clinical or in preventive medicine or in fundamental research. As will be shown, simplicity, speed and moderate assay cost may well, at least in some situations, trade off for less-than-optimal sensitivity or analytical precision.

2. Assay of methotrexate and its metabolites by liquid chromatography

The essential experimental characteristics of a number of high-performance liquid chromatography methods employed for the measurement of MTX and its metabolites, OHM and DAMPA are collected in Tables 1–3. Table 1 reports details of 21 assays to measure only MTX [14–30]. Table 2 reports details of 34 assays to measure both MTX and OHM [31–63]. Table 3 reports details of 12 assays to measure MTX, OHM and DAMPA [64–75] and of a further five methods, some of which also separate some relevant folates, such as 5-methyl- and 5-formyl-tetrahydrofolate (leucovorin) [76–80] in clinical samples.

The 67 papers considered have been extracted from a database of more than 200 references published in the years from 1975 to 2000. The literature was searched on the Medline and the Chemical Abstract reference collections, and selected mainly on the basis of the completeness of the reported analytical information to the aim of reproducing the assay. A few papers report alternative variations to the general analytical procedure described, e.g. the use of different sample preparation techniques or chromatographic columns.

As apparent from the large amount of missing (nr, not reported) information in the tables, often papers published in biomedical rather than analytical journals did not report precisely some important details of the assays employed, mainly regarding their quantitative performance (recovery, detection limits and linearity range, precision and accuracy), but not infrequently even on experimental variables, such as the detailed characteristics of the chromatographic column and of mobile phase composition.

According to their chemical properties, MTX, its metabolites and analogue drugs present in several biological samples can be extracted, separated and detected under a variety of chromatographic conditions, i.e. on different stationary phases, under a wide choice of mobile phase conditions, followed by several detection techniques. Simpler — yet highly specific and sensitive — methods are required to detect MTX in environmental samples such as air and surface particulates and sewage waters.

2.1. Sample preparation

The choice of optimal techniques for pre-chromatographic sample preparation is a much addressed problem in the set-up of analytical methods for the measurement of MTX in biological samples, mainly in the field of therapeutic drug concentration monitoring (TDM), but also in large clinical pharmacokinetic (cPK) studies, since this is the most labour-intensive, rate-limiting and often expensive step of the entire analytical procedure. Sample preparation is also the step of the analytical procedure which most strictly depends on the nature of the biological matrix: the concentration of MTX and its metabolites has been measured under different circumstances in plasma, serum, erythrocite lysates, cerebrospinal fluid, urine and tissue homogenates, as well as in some environmental samples, such as wipe samples from surfaces, airborne particulates collected on filters and sewage water.

2.1.1. Direct injection

Direct injection of plasma or serum samples into the chromatograph is — of course — the simplest approach in TDM and cPK, essentially requiring no sample preparation besides whole blood separation. Plasma proteins, which constitute the main sample matrix, can be eluted in the column void volume only at neutral pH, otherwise they would precipitate and clog the column, and thus the requisite of direct plasma injection determines a boundary condition for the separation method. Only a few papers (9/67) [16,22,29,32,36,69,73,74,76] report direct serum or sample injection, without on-line sample preparation techniques, and nearly always employ a precolumn to protect the analytical column from a fast loss of efficiency. The pH of the mobile phase is, in all

except three methods, buffered at values of 6.2–7.5. In a single case [22], proteins are kept in solution with the use of a high concentration of a detergent, so that a weakly acidic (pH 5.7) mobile phase could be used, while in other cases [69,76] a frequent change of the pre-column allowed the use of a much more acidic (pH 3.2–3.6) eluant.

2.1.2. Protein precipitation

This is a fast, simple and inexpensive preparation method for most biological fluids, which entails deproteinization of plasma, serum or tissue samples with strong acids: trichloroacetic [18,20,24,46,49, 70,75] and perchloric [38,61] acid, or with water-miscible organic solvents such as acetonitrile [43], followed by a fast centrifugation to separate the protein pellet and by injection into the chromatograph of an aliquot of the supernatant.

However there are two drawbacks to this sample preparation technique: first, late-eluting lipid components of plasma are not eluted, and thus accumulate in the column at each injection, deteriorating its separation efficiency over time. Second, when an organic solvent is employed as the protein precipitant and an aliquot of the supernatant is directly injected, the analytes are dissolved in a strong eluant, and injection of a ''plug'' of it into a much weaker mobile phase determines a loss of separating efficiency of the reversed-phase column, the influence of which is more pronounced as progressively shorter columns are employed.

These drawbacks can, however, be overcome, since MTX and its metabolites are rather hydrophilic analytes. In fact, further sample cleanup can be achieved through extraction of the centrifuged sample with an appropriate organic solvent: the fairly large volume of organic solvent employed (up to fivefold the sample volume) back-extracts the organic solvent employed for protein precipitation and some of the water in the sample, while leaving a smaller water volume (in the order of 100 μl), which can be directly injected into the system. This apparently simple modification of the procedure allows, within a single fast step and without relevant influence on the analyte recovery, to improve the chromatographic separation through elimination of the strong solvent, to inject a concentrated sample in a less hydrophobic solvent and also to extend column lifetime by getting rid of the lipid component of plasma.

One method employed to lower the concentration of acetonitrile employed to precipitate the proteins in the aqueous phase to be injected was ice-cooling the solution [21].

Among the most commonly employed solvents employed for back-extracting the precipitating acetonitrile [33,67] or acetone [27,45,49,59], one is a *n*-butanol-diethyl ether mixture (approx. 3:4 v/v) first proposed by Brimmel and Sams. Another solvent mixture for back-extracting acetonitrile is a 10:1 ethyl acetate/*iso*-amyl alcohol mixture [66]. In both cases, the small water volume to be injected is on the bottom of the extraction tube. A more recent alternative is the use of acetonitrile as the protein precipitant and of chloroform to back-extract the organic solvent [52,58]. In this case, the water volume containing the analytes is on top of the organic solvent layer.

2.1.3. Liquid–liquid extraction

Although MTX and its metabolites are poorly soluble molecules, precipitation of plasma proteins, either by strong acids or with organic solvents, allows to extract the aqueous supernatant with immiscible solvents, which can be separated, taken to dryness and the residue reconstituted for injection into the liquid chromatograph in a suitable volume of mobile phase or of a compatible solvent. The most frequently employed procedure uses perchloric [31,37] acid to precipitate proteins and extraction with a 10:1 mixture of ethyl acetate and iso-propanol. Variants to the base procedure include neutralization with potassium hydroxide of the acidic supernatant before extraction [64], protein precipitation with sulfuric acid/ammonium sulfate [23], or with trichloroacetic acid in ethanol and extraction with ethyl acetate [71].

Of course, a major disadvantage of sample preparation procedures entailing liquid–liquid extraction is that fairly large volumes of high-purity organic solvents are employed and either concentrated by evaporation or discarded. High-purity organic solvents are quite expensive and often subject to regulations and evaporation of relatively large quantities of volatile solvents, in the order of 50–500 ml

Table 4
Experimental conditions for the solid-phase extraction of methotrexate and some metabolites from biological fluids and environmental matrices

Sample	Volume (ml)	Sorbent	Column conditioning	Sample loading	Interference elution	Analyte eleution	Pre-injection treatment	Injected volume (μl)	Ref.
P	1	BondElute C_{18}	1 ml MeOH 1.5 ml 0.05 *M* PB pH 2.7	1 ml sample+ 1 ml 0.05 *M* PB pH 6.5 (2 ml/min)	2 ml 0.5 *M* PB pH 2.7 1 ml 0.1 *M* NaOH 1 ml 0.5 *M* PB pH 2.7	1.5 ml MeOH	Evap. diss. in mobile phase (0.2 ml)	100/200	[53]
P	1	BondElute C_{18}	2.5 ml MeOH 2.5 ml 0.1 *M* H_3PO_4	1 ml sample+ 0.05 ml IS+ 1 ml 0.1 *M* H_3PO_4	2 ml (5% MeOH in 0.1 *M* H_3PO_4) 2 ml 0.1 *M* PB pH 8 2.5 ml (H_2O/MeOH 3:1)	1 ml (MeOH/ HCHO 98:2)	Evap. diss in 0.2 ml 0.1 *M* H_3PO_4	200/200	[60]
P	0.5	C_{18}	Not rep.	0.5 ml sample+ 0.5 ml (10 g/l)ascorbic acid+0.01 ml IS+ 0.5 ml 5% AcOH	2 ml 10 m*M* AFB pH 3.5 1 g/l ascorbic acid	95% (10 m*M* PB– 1 g/l ascorbic acid)–5% ACN pH 6	None	nr	[79]
P	0.01–1	Isolute HAX 200	3 ml MeOH+ 3 ml 0.1 *M* H_3PO_4	0.1–1 ml sample 2 ml 0.1 *M* H_3PO_4 (flow 2.5 ml/min)	2 ml (95% 0.1 *M* H_3PO_4 4.5% MeOH) 3 ml NaH_2PO_4 pH 8.6	2 ml (20 g/l TFA in MeOH)	Evap. diss. in 0.1 ml 0.1 *M* H_3PO_4	20/100	[54]
P	2	Sep-Pak C_{18}	10 ml MeOH 10 ml H_2O 10 ml 0.02 *M* AcB pH 5.5	Apply to column supernatant of: 1.920 ml sample+ 0.08 ml IS+0.2 ml (35% $HClO_4$)	10 ml H_2O 2 ml(H_2O/MeOH 9:1)	MeOH	Evap. diss. in 0.1 ml 0.005 *M* HCl	100	[47]
P, CSF	1–2	PRE-SEP 1 C_{18}	2 ml MeOH 2 ml H_2O 2 ml PB pH 6.8	1–2 ml sample	2 ml PB air dry	2 ml MeOH	Evap. diss. in mobile phase	nr	[48]
P, S	1	Sep-Pak C_{18}	10 ml MeOH 10 ml 0.05 *M* AcB pH 5.5	1 ml sample+ 2 ml H_2O+ 2 ml 0.01 *M* Tris	10 ml AcB pH 5.5 2 ml 0.1 *M* NaOH 3 ml AcB pH 5.5	2 ml MeOH	Evap. diss. in mobile phase	20/100	[24]
P, sal, U	1 (P) 0.1 (sal) 0.01 (U)	Isolute C_8	3 ml MeOH+ 3 ml 0.1 *M* H_3PO_4 (flow 2.5 ml/min)	Sample+ 2 ml 0.1 *M* H_3PO_4	2 ml (95% H_2O:5% MeOH)	2 ml (MeOH/ TFA 98:2)	Evap. diss. in 0.1 ml H_2O	10/100	[72]
P, U	3	AG-X2	10 ml H_2O 2 ml 1 N AcOH	3 ml sample	Effluent discarded	6 ml 2 N AcOH	Evap. diss. in mobile phase	nr	[78]
P, U	1	SepPak Certify C_{18}	2.5 ml MeOH 2.5 ml 0.1 *M* H_3PO_4	1 ml sample (0.01 ml for urine)+ 1 ml 0.1 *M* H_3PO_4 (2 ml/min)	2 ml (95% 0.1 *M* H_3PO_4 4.5% MeOH) 2 ml 0.1 *M* NaH_2PO_4 pH 8 2.5 ml (H_2O/MeOH 75:25)	1 ml (MeOH/ HCHO 98:2)	Evap. diss. 0.1 *M* H_3PO_4 (0.5 ml plasma extr. 1 ml urine extr.)	100/500	[50]
S	0.2	SepPak	10 ml MeOH 10 ml 0.2 *M* AcB pH 5.5	0.2 ml sample+ 0.2 ml IS+ 0.4 ml 10 m*M* Tris pH 5.5	5 ml AcB 1 ml 0.1 NaOH 1 ml AcB	2 ml MeOH	Evap. diss. in mobile phase	80/100	[35]
S	0.2	SepPak C_{18}	10 ml H_2O 10 ml 0.2 *M* AcB 5.5	0.2 ml sample+ 0.4 ml 0.01 *M* tromethamine pH 9 1 ml H_2O	5 ml AcB 1 ml 0.1 *M* NaOH 1 ml AcB	2 ml MeOH	Evap. diss. in mobile phase	nr	[39]

Table 4. Continued

Sample	Volume (ml)	Sorbent	Column conditioning	Sample loading	Interference elution	Analyte eleution	Pre-injection treatment	Injected volume (μl)	Ref.
S	0.3	SepPak C_{18}	10 ml H_2O 10 ml 0.2 *M* AcB pH 5.0	0.3 ml sample+ 5 ml 0.2 *M* AcB pH 5.0	10 ml H_2O	2 ml MeOH	Evap. diss. in 0.005 *M* HCl	75/200	[40]
S	1	SepPak C_{18}	10 ml MeOH 10 ml H_2O	1 ml sample	5 ml H_2O	3 ml H_2O	Evap. diss. in 100 μl (H_2O/MeOH 80:20)	20/100	[41]
S	0.5	BondElut C_{18} 200 mg	5 ml MeOH 3 ml (0.05 *M* citric acid-0.1 *M* NaH_2HPO_4 pH 5	0.5 ml sample+ 2.5 ml (0.1 *M* citric acid–0.2 *M* Na_2HPO_4 pH 5	3×1 ml B 0.5 ml 0.02 *M* NaOH 0.5 ml B	1 ml MeOH	Evap. diss. in 0.1 ml Tris–HCl	20/100	[77]
S	nr	SepPak C_{18}	nr	Centrifuged supernatant of: serum sample+$HClO_4$ sol. applied to column	nr	Not rep.	nr	nr	[51]
S	0.2–0.5	SepPak Classic C_{18}	20 ml MeOH 3 ml 0.2 *M* PB pH 6	0.2–0.5 ml sample+1 ml IS	1 ml H_2O	2 ml MeOH	Evap. diss. in 0.2–0.3 ml 0.005 *M* HCl		[55]
S, U, bile	1	BondElute C_{18}	nr	1 ml sample+ 0.1–1 ml IS+ 1–2 ml 0.01 *M* Tris pH 7	10 ml 0.056 *M* PB pH 2.7 2 ml 0.1 *M* NaOH 3 ml 0.056 *M* PB pH 2.7	3 ml MeOH	Evap. diss. in 250 μl mobile phase	50/250	[68]
U	1	BondElute Phenyl	1 ml MeOH 1 ml H_2O	1 ml sample	2 ml H_2O 1 ml AcOEt	2 ml MeOH	Evap. diss. in H_2O	10–50/100	[30]
U	1	Amberlite	None	1 ml sample+ 1 ml 0.1 *M* Tris	10 ml H_2O 4 ml MeOH	6 ml (MeOH/AcOEt 3:1)	Evap. diss. in 0.1–1 ml mobile phase	20/100	[24]
U	1	SepPak silica	None	1 ml sample+0.25 ml 0.1 *M* AcB pH 5, 0.25 ml of mix. loaded in cartridge.	5 ml AcOEt 5 ml (EtOH/MeOH 6:4)	3 ml (1%NH_3 in MeOH)	Evap. diss. in mobile phase (0.1 ml)	20/100	[57]
U	5	Isolute C_{18}	6 ml MeOH 6 ml 0.002 *M* NH_4OAc pH 4	5 ml sample+ 5 ml 0.02 *M* NH_4OAc pH 4 (1 ml/min)	6 ml AcOEt	3×1 ml MeOH	Evap. diss. in mobile phase	10/200	[62]
Sewage water	1000	XAD-4	20 ml 0.01 *M* KH_2PO_4 pH 3 20 ml 0.2 *M* HCl	1000 ml sample at pH 3.0 (5 ml/min)	1 ml H_2O	4×1 ml MeOH	Evap. to 1 ml	20	[80]
Sewage water	1000	XAD-7	10 ml 0.01 *M* KH_2PO_4 pH 3 20 ml 0.2 *M* HCl	1000 ml sample at pH 3.0 (5 ml/min)	None	4×1 ml MeOH	Evap. to 1 ml	20	[80]
Sewage water	1000	C_8 and C_{18} different brands	2 ml MeOH 3 ml 0.1 *M* KH_2PO_4 pH 6	1000 ml sample at pH 3.0 (5 ml/min)	1 ml H_2O	3–5×1 ml MeOH	Evap. to 1 ml	20	[80]
Wipe samples	20–100	BondElute Jr C_{18}	3 ml MeOH 3 ml H_2O cartridge drying	20–100 ml	Effluent discarded cartridge drying	2×1 ml (1% NH_3 in MeOH)	Evap. diss. in 1 ml MeOH	20/1000	[28]
Wipe samples	10	Isolute C_{18}	6 ml MeOH 6 ml 0.002 *M* NH_4OAc pH 4	10 ml sample adjusted at pH 4 (1 ml/min)	None	3×1 ml MeOH	Evap. diss. in mobile phase	10/200	[63]

Abbreviations: AcB, acetate buffer (acetic acid–Na acetate); CSF, cerebro-spinal fluid; IS, internal standard; NH_4OAc, ammonium acetate; nr, not reported; P, plasma; PB, phosphate buffer; S, serum; sal, saliva; U, urine.

per day, often poses an occupational hazard, due to their toxicity and fire hazard.

2.1.4. Off-line solid-phase extraction

Off-line solid-phase extraction (SPE) of plasma or urine is a more elaborate and expensive preparation technique to clean and to concentrate the sample. Several parameters can be modulated by the analyst to achieve an efficient pre-purification of the sample, ranging from the nature of the employed sorbent to the conditions for cartridge preconditioning, sample loading, contaminant elution and analyte recovery. Table 4 collects the experimental conditions described in 26 papers that employ SPE for the extraction of MTX, OHM and DAMPA from matrices such as serum, plasma and urine.

As shown, different sorbents have been employed, acting by ion-exchange, direct- or reversed-phase mechanism, the last one being the most commonly employed. Since MTX is poorly retained on reversed-phase media, in all but one case, the most lipophilic sorbent, i.e. octadecyl-derivatized silica is employed. Conditions for cartridge conditioning, sample loading, cartridge washing and analyte elution vary greatly among the different methods published: in particular, plasma and serum are either loaded as such or diluted in an acidic solution. Interference elution is achieved by washing the cartridge with acidic, neutral or alkaline solutions or with organic solvents, while analyte elution is performed with organic solvents (most often methanol, in one case acetonitrile), frequently mixed to strong organic acids.

2.1.5. On-line sample preparation

On-line sample preparation is the final step in thorough automation of the analytical method. This approach requires substantially sophisticated and expensive instrumentation and highly skilled technicians to allow its routine use. So far, a few such methods have been published [15,19,26,30,34,56], which employ substantially different approaches.

Lankelma and Poppe [15] describe the coupling of a pre-concentration column packed with a C_8 alkyl-silica reversed-phase sorbent to an analytical column packed with SAX (silica-bound strong anion-exchanger) material. A 1-ml portion of the supernatant from TCA–HCl protein precipitation of a 1-ml plasma sample is fed to the pre-concentration column, which is further washed with 20 ml of pure water to elute the poorly retained sample contaminants. The analyte MTX is loaded on to the separation column by back-flushing with the chromatographic eluent at a flow-rate of approx. 2.4 ml/min. MTX elution under these conditions is achieved in approx. 5 min. The technique was further adapted to the simultaneous determination of MTX and OHM, yielding one of the fastest described analytical methods [34].

The opposite strategy in column coupling was followed by Mader et al. [30], who developed a very sensitive method for the measurement of trace MTX in the urine of hospital nurses administering high-dose MTX chemotherapy to osteosarcoma patients. The sample injected in the chromatographic system was the eluate from the C_{18} SPE of a 1-ml urine sample. The concentration pre-column was a strong anion-exchange column (Nucleosil 100SB), coupled to a C_{18} reversed-phase analytical column. MTX was eluted from the anion-exchange column with a ionization-suppressing acidic buffer containing 1% acetonitrile, and the heart-cut eluate (between 9.5 and 14.5 min, with a mean retention time of MTX of 11–12 min) loaded on to the RP column. Under such a low proportion of acetonitrile in the acidic mobile phase, MTX is concentrated on the head of the RP column, whence it is eluted as a very sharp peak with a gradient of acetonitrile. The retention time of MTX globally resulting from this procedure is approx. 35 min.

Direct injection of plasma samples by column-switching was accomplished by coupling a preconcentration and an analytical column packed with an octadecyl-silica sorbent and stationary phase [17]. MTX in the injected plasma was retained in the precolumn, while plasma proteins and interferents were washed to the waste with a neutral phosphate buffer. The heart-cut fraction containing MTX was subsequently loaded from the preconcentration column on to the analytical column with a phosphate/acetonitrile gradient and finally eluted from the analytical column in the isocratic mode, by ion-pairing with tetrabutyl-ammonium. The sample cycle time was 40 min.

Another approach for direct injection of plasma samples by column-switching is described in two

recent papers [19,56], in which the analytical methods differ only in the detection technique, which is UV–Vis spectrophotometry in the former, post-column oxidation and fluorimetry in the latter. The sorbent in the pre-concentration column is an alkyl-diol silica C_8, while the analytical column is a conventional RP-18 one. A 100-μl plasma sample was injected on the precolumn and the plasma proteins discarded by flushing the column with a neutral buffer containing 1% acetonitrile as the organic modifier and tetrabutyl-ammonium as the ion-pairing reagent. Under these conditions, the lipophilic ion pair between MTX (or its metabolite, OHM) and the organic cation is strongly retained on to the pre-column and can be transferred on to the analytical column by back-flushing the precolumn with the separation mobile phase, which contains a substantially higher (18%) proportion of acetonitrile. This method allowed a global sample analysis time of 16 min. Critical parameters in the adjustment of the retention of the analytes on the preconcentration sorbent were both the concentration of the tetrabutyl-ammonium ion-pairing reagent and that of the organic modifier.

An ingenious coupling of three columns has been employed to measure MTX in plasma samples with an automated technique, involving fluorimetric detection. The assay entails on-line sample preparation by pre-chromatographic oxidation of MTX to the corresponding pterine-carboxylic acid by a Ce(IV) trihydroxyhydroperoxide coordination compound immobilized in a column flow reactor, separation of the fluorescent analyte from reaction byproducts on a protein-coated precolumn and subsequent isocratic elution from an octadecyl-silica analytical column [26]. The global analysis time is of 15 min per sample and the lower limit of detection of the assay is in the 100 n*M* range.

2.2. Chromatographic conditions

Of the 70 assays considered, 21 measure only MTX, 34 measure both MTX and OHM, 12 measure all three main metabolites: MTX, OHM and DAMPA and three also measure leucovorin and 5-methyl-tetrahydrofolate. MTX and its metabolites can be separated under a wide variety of different conditions, by reversed-phase, ion-pairing or ion-exchange chromatography.

2.2.1. Reverse-phase separation

By far the most frequently employed stationary phase employed under reversed-phase and ion-pairing conditions is the C_{18} alkyl-silica, although a few exceptions have been proposed: two reports describe the use of a C_8 phenyl column [33,67], one of a custom-prepared column packed with ODS-derivatized porous glass beads [27]. Polystyrene–divinylbenzene (PRP-1) has also been employed as the stationary phase due to its much higher resistance to the strongly oxidizing mobile phase employed for post-column photoxidation prior to fluorimetric detection (vide infra) [60].

Under reversed-phase chromatography conditions on octadecyl-silica columns, MTX and its metabolites can be eluted under isocratic conditions by employing mixtures of aqueous buffers at pH values ranging from 2.5 to 6.7 with several organic solvents, the most commonly employed being acetonitrile (19 papers/36), methanol (12 papers/36) and — to a much lesser extent — also tetrahydrofuran (three papers/36) and dimethylformamide (two papers/36). In a few cases (nine papers/36), mixtures of organic solvents, such as methanol and acetonitrile [20,21,24,35,39,42,71], methanol and tetrahydrofuran [69] or acetonitrile and dimethylformamide [53] have been employed to improve the separation.

The length of the isocratic analytical runs varies according to the number of the targeted analytes (MTX alone, MTX and OHM or MTX, OHM and DAMPA) and to the selected instrumental conditions, depending also on the degree of pre-purification of the injected samples and on the specificity of the employed detection system. The reported analysis time for biological samples varies between 8 and 40 min. The length of chromatographic runs able to separate all three target analytes may be as short as 8 min, using a pH 3 citric acid–phosphate buffer, modified with a mixture of methanol and tetrahydrofuran [69].

In one paper, a mobile phase containing a high concentration of sodium dodecylsulfate at mildly acidic pH is employed to keep plasma proteins in solution, thus allowing the direct injection of plasma

samples on to the analytical column and the measurement of MTX within 7 min [22].

Only a few methods employ gradient elution for the separation of MTX [29,30], its metabolites [51,75,80] and some reduced folates [77,79], but since this technique is inherently slower, due to the necessity to re-equilibrate the column with the starting mobile phase before each run, it is generally less suitable for routine analytical use, unless complex multicomponent separations are needed.

2.2.2. Ion-pairing chromatography

Methotrexate is a hydrophilic poly-electrolyte which can exist as deprotonated, zwitter-ionic or protonated molecules according to solution pH. Since its retention on the octadecyl-silica stationary phase is relatively poor, ion pairing with hydrophobic anions or cations can be employed to enhance its retention. Ion-pairing with tetrabutyl-ammonium reagents under neutral buffer conditions [19,25, 32,36,56] or with tetramethyl-ammonium [55] or hexanesulphonic acid [64] under acidic conditions have been employed, although not greatly improving the separation, when compared to other methods with a similar duration of the chromatographic run. Ion-pairing with tetrabutyl-ammonium has been employed to retain MTX on a C_8 alkyl-diol silica precolumn in a method which entails on-line sample preparation [19,56].

In contrast, a field where the use of ion-pairing chromatography yields impressive improvement of the separations is the determination of the intracellular polyglutamate active metabolites of the natural folates and of MTX, which will be discussed in Section 4.

2.2.3. Ion-exchange chromatography

This separation technique has been comparatively little exploited, in comparison to reversed-phase separation. Due to its amphoteric poly-electrolyte nature, MTX can be separated both by cation- [64,66] and anion- [14,15,31,34] exchange. In particular, cation-exchange demonstrated unique performance, allowing the elution of OHM, MTX and DAMPA in plasma, saliva and urine samples within 10 min by employing a SAX™ (silica-based alkyl-trimethylammonium) strong anion-exchange column [66].

Coupling of on-line sample concentration on a C_8 precolumn and analytical separation on a SAX™ column yields one of the fastest analytical methods, enabling simultaneous measurement of MTX and OHM in plasma samples within 10 min [34].

2.2.4. Retention behaviour of MTX and related compounds

In a few papers, the authors report data on the variation of the retention of some analytes (most frequently MTX and OHM, in a few cases also DAMPA and AMP) on C_{18} reversed-phase columns as a function of the composition [19,28,56,69] or pH [28,57,74,81] of the mobile phase. Since the data were obtained under very different experimental conditions (mainly of column length and mobile phase composition), only limited general considerations can be drawn on the influence of the individual parameters on chromatographic separation. In general terms, the lower the pH, the longer is the retention of all the analytes on the column, but the separation factor decreases [28,57]. As for the effect of the different organic solvents as mobile phase modifiers, in particular, the use of a 4:1 methanol/tetrahydrofuran mixture in a phosphate–citrate acidic buffer lends impressive benefit in hastening the elution of the late-eluting DAMPA [69].

Table 5 reports a range of retention time and capacity factor (k') values of MTX and of separation factors of OHM, DAMPA and AMP relative to MTX, necessary for the separation of the target analytes in 50 assays where chromatographic separation was accomplished on octadecyl-silica columns under isocratic elution conditions and without ion-pairing reagents. The values have been calculated — when possible — on the basis of the chromatographic data reported in the considered papers. In 11 cases, only MTX was measured, in 18 cases the assay determined both MTX and OHM, while in six cases MTX, OHM and DAMPA were separated.

Under such conditions, the k' of MTX ranges between 0.3 and 21.3 with a median value of 2.8–3.3 depending on the complexity of the separation; that of OHM ranges between 0.5 and 15.8 with a median value of 4.0–5.8 and that of DAMPA between 1.5 and 21.2 with a median value of 10.

As expected, the des-methyl analogue aminopterin (AMP) elutes much earlier than MTX under re-

Table 5
Range of retention time, capacity factor and separation factor values for separations of methotrexate and its metabolites on octadecyl-silica columns under isocratic conditions

Separation		$t_{r\,MTX}$	k'_{MTX}	k'_{OHM}	k'_{DAMPA}	k'_{AMP}	α_{OHM}	α_{DAMPA}	α_{AMP}
MTX (n=11)	Min	2.5	0.7			0.2			0.2
	Median	10.0	3.2			2.5			0.2
	Max	15.0	21.3			4.7			0.5
MTX+OHM	Min	0.6	0.3	1.0		2.7	1.21		0.47
(n=18)	Median	7.5	2.8	4.0			1.39		
	Max	42.0	12.9	15.8			3.68		
MTX+OHM+DAMPA	Min	5.5	0.4	0.5	1.5	5.1	1.20	1.36	0.69
(n=6)	Median	6.5	3.3	5.8	10.0		1.58	3.40	
	Max	7.5	9.6	14.5	21.2		2.42	5.23	

t_r, retention time; k', capacity factor; α, separation factor.

versed-phase chromatography conditions, with a value for the α separation factor between 0.2 and 0.7. The metabolite DAMPA elutes much later than MTX, with a median value of the separation factor higher than three. This also is an expected behaviour, since the compound lacks the glutamic acid portion and is consequently much less polar than MTX; as a consequence, the analysis time is much longer if this metabolite has also to be measured in samples. To overcome this difficulty, one paper reports the use of an acidic mobile phase containing citric acid and tetrahydrofuran which allows to reduce the capacity factor of DAMPA to less than half, thus allowing the separation of all three analytes, MTX, OHM and DAMPA in less than 10 min.

Under reversed-phase conditions, the metabolite OHM elutes after the parent drug MTX, with separation factors relative to MTX between 1.2 and 2.4. In contrast, under cation-exchange conditions, OHM elutes before MTX ($0.6<\alpha<0.8$), thus suggesting a decrease in the acidity of the carboxyl groups, with respect to MTX.

The observation that the hydroxylated drug metabolite elutes later than the parent drug is a surprising finding, as long as it deviates from the widely accepted paradigm which suggests that drug metabolization by hydroxylation generally increases polarity and hydrophilicity with respect to the parent compound. The modification of the chemico-physical characteristics of the hydroxylated metabolite with respect to the parent molecule is possibly a consequence of the interaction of the 7-hydroxyl group with the other polar groups in the molecule, the N^{10} amino function and the carboxyl groups, and results also in a lower solubility of OHM with respect to MTX, especially in acidic solution.

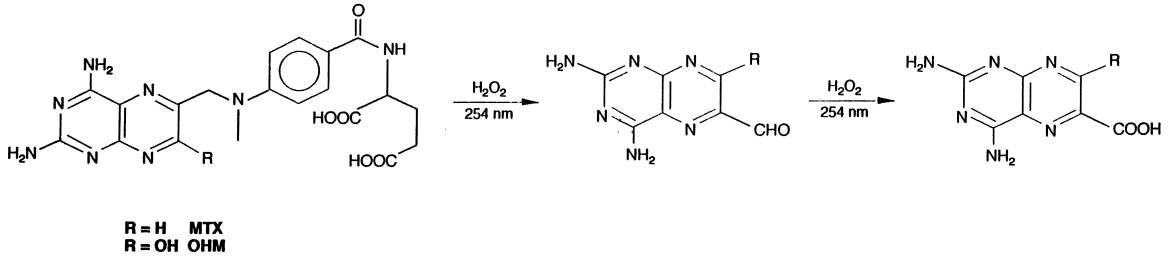


Fig. 5. Proposed mechanism of the photo-oxidation of methotrexate and 7-OH-methotrexate to fluorescent pterine-carboxylic acids.

2.3. Detection techniques

MTX and its metabolites can be detected with most chromatographic detectors: UV–Vis spectrophotometry, fluorimetry, electrochemical oxidation and mass spectrometry, the first one being the most frequently employed.

2.3.1. UV–Visible spectrophotometry

UV spectrophotometry is the most commonly employed technique for the determination of MTX, since 50/72 of the considered assays use this technique. MTX is a strongly UV–Vis absorbing substance, due to the presence of the heteroaromatic pterine chromophore, and the absorbance maxima

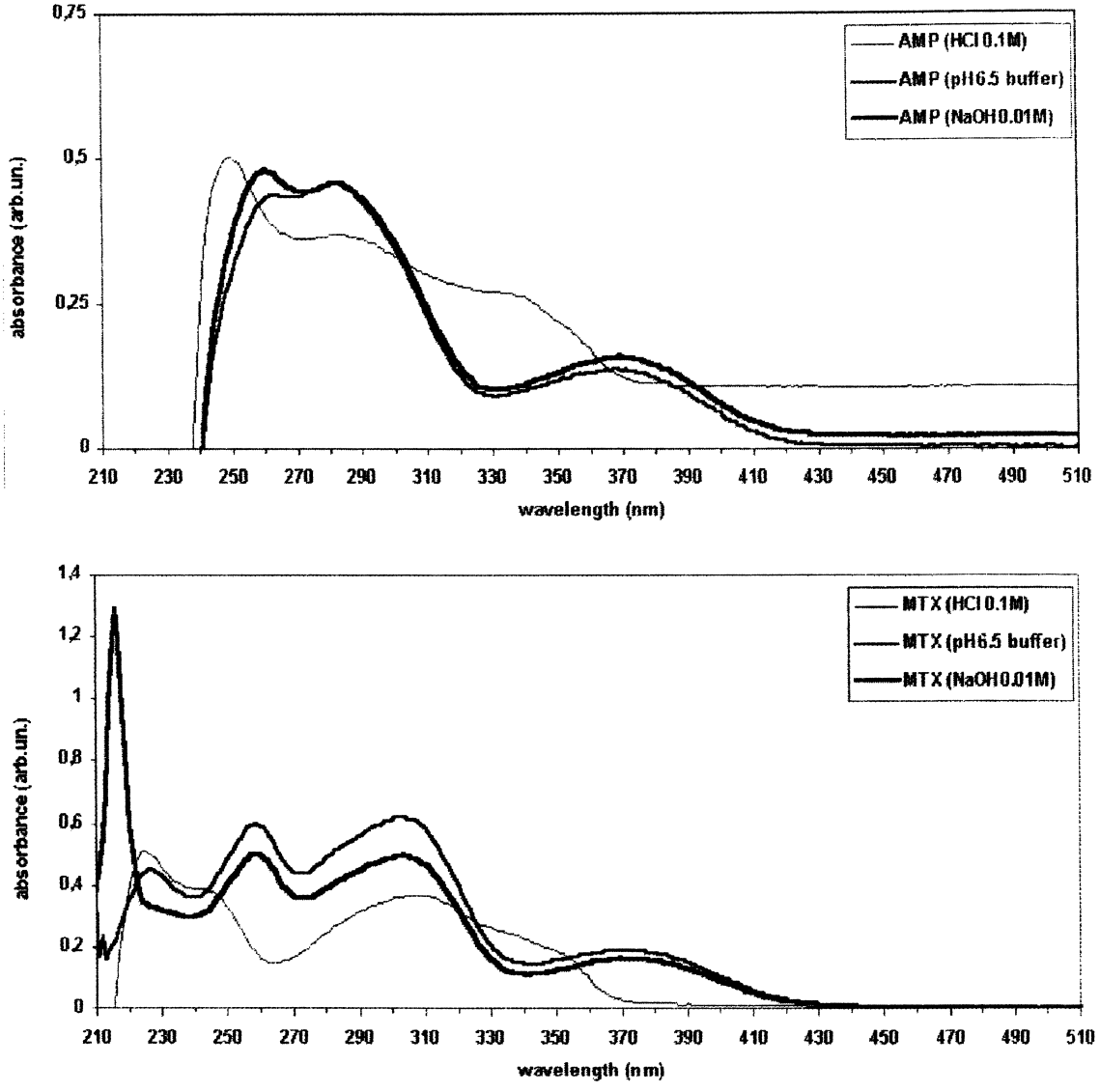


Fig. 6. Electronic spectra of aminopterin (upper box) and methotrexate (lower box) at three different pH values: 1 (0.1 *M* HCl), 6.5 (100 m*M* NH_4OAc buffer) and 8 (0.01 *M* NaOH).

are strongly dependent on mobile phase pH, as shown in the spectra of Fig. 6. The highest molar extinction of the pterine chromophore is obtained at neutral pH, while at acidic pH absorbance is reduced by at least a factor of two.

The most commonly employed wavelength is between 303 and 313 nm (42/50 assays), with five papers reporting the use of the much less specific wavelength of 254 nm, two reporting the use of a wavelength at 370 nm and two that of diode-array detection [22,80].

The lower limit of detection achieved for MTX ranges between 20 and 200 n*M* in the biological sample, depending on the degree of sample enrichment achieved during preparation.

2.3.2. Fluorimetry

Fluorimetry is employed as a very specific and the most sensitive HPLC detection technique for MTX, besides tandem mass spectrometry, since it allows to attain sensitivity levels down to the low-ng range (corresponding to a few picomol) of injected analytes.

While some natural folates are fluorescent per se, MTX and its metabolites are not; fluorescent products are the pterine-carboxylic acids arising from oxidative degradation of MTX, of OHM and of DAMPA. These products can be generated either before or after the chromatographic separation.

Pre-chromatographic oxidation of the analyte(s) can be achieved directly in the plasma sample with potassium permanganate and quenching the excess oxidant with H_2O_2. The oxidation products are chromatographically separated and detected by fluorimetry [18,25]. More recently, on-line Ce(IV) oxidation of serum samples in a pre-column flow-reactor has been employed, followed by on-line sample pre-purification and fluorimetric detection [26]. One disadvantage of pre-column oxidation is that DAMPA yields the same oxidation product of native MTX, thus potentially leading to overestimation of the concentration of the parent drug.

Post-chromatographic oxidation is performed by photo-oxidation, with UV irradiation at neutral pH in the presence of H_2O_2 [50,53,54,56,60,72–74], according to the reaction scheme in Fig. 5. The reaction is performed in a post-column flow photo-reactor made up of a transparent polymeric tubing coiled around a fluorescent tube lamp irradiating UV light at 254 nm. Detection of the specific oxidation products is accomplished by exciting with light at 350–370 nm and detecting the emission at 417–465 nm. Concentration of H_2O_2, nature and concentration of the organic modifiers in the mobile phase, reaction time in the coil are the parameters playing a substantial role in achieving the maximum detection sensitivity, which is 50- to 100-fold higher than that achieved by UV–Vis detection. Usually, DMF is incorporated into the mobile phase, either as the sole organic modifier or in a mixture with acetonitrile and the mobile phase mixture is degassed before use or aged overnight to eliminate dissolved oxygen, which has a detrimental effect on development of fluorescence. The most convenient tubing material was found to be tefzel and the diameter and length of the coil are usually set so as to allow a permanence or reaction time in the photo-reactor of 0.05–0.7 min.

The use of fluorimetric detection restricts the chromatographic conditions to isocratic elution at neutral pH (6.2–6.7) and dictates for the use of a mobile phase containing hydrogen peroxide (0.1–0.5%) and commonly of dimethylformamide as organic modifier. Of course, the use of such a strongly aggressive mobile phase has consequences on the durability of the alkyl-silica stationary phase of the column, as highlighted by the observation of a black residue on top of the column packing. To overcome this problem, the authors employed an alternative packing material as the stationary phase: an automated analysis method with post-column oxidation and fluorescence detection is described, where separation of MTX and its metabolites is accomplished on a polystyrene–divinylbenzene (PRP-1) column, since this stationary phase is more resistant than alkyl-silica to deterioration with the strongly oxidizing mobile phase employed [60].

2.3.3. Electrochemical detection

Although the substituted pterine ring is highly electroactive and electrochemical detection has been widely applied to the HPLC determination of biological folates, only two papers employ this technique for the measurement of MTX [23,24].

The first report of electrochemical detection to measure serum MTX with HPLC involved the use of a dual coulometric detector, whereby a first step of

oxidation was performed at +0.5 V to get rid of the bulk of the eluting interferent and a second step at +1.1 V was employed for detection. Under the established conditions, and employing *p*-amino-acetophenone (pAAP) as the internal standard, the lowest measurable concentration of MTX was 22 μ*M* [23].

Later, Palmisano et al. performed a systematic study on the anodic oxidation of MTX and reported that the analyte undergoes a two-electron oxidation process at +0.95 V versus SCE, possibly leading to production of the 7-hydroxy-MTX metabolite. The described analytical method employs theophylline as an electro-active internal standard and allows to detect 50–500 ng of MTX on-column within a 20-min chromatographic run [24].

2.3.4. Mass spectrometry

Mass spectrometric techniques, although long employed for structural purposes [82,83], could not be applied to the measurement of intact glutamyl-pterines, such as MTX, due to their high polarity, consequent low volatility and thermal instability, until the availability of modern techniques, such as electrospray-ionspray, which allowed to couple mass spectrometric detection to liquid chromatography.

In fact, only Przybylsky et al. [84] reported an early attempt to identify MTX metabolites (OHM and DAMPA) and to quantify MTX and OHM in patient's urine by off-line analysis with field-desorption mass spectrometry of chromatographic fractions collected from a custom-designed HPLC run, which employs a volatile ammonium bicarbonate buffer instead of the commonly employed phosphate.

So far, two methods for the measurement by HPLC–MS–MS of MTX and OHM have been published. One [58] is aimed at measuring the target analytes MTX and OHM at plasma concentrations down to 100 and 300 pg/ml, respectively, with a very efficient use of costly mass spectrometer working time. To achieve this goal, plasma samples were processed off-line by acetonitrile protein precipitation and liquid–liquid extraction with chloroform. In order to load a large volume (80 μl) of the reconstituted extracts into the HPLC–MS–MS system equipped with a narrow-bore (1 mm I.D.) HPLC column operating at a flow of 0.15 ml/min, a trapping pre-column was employed. The analytes were eluted into the electrospray ion source and ionized to the corresponding protonated molecules $[MH]^+$. Selective detection was accomplished by selected reaction monitoring on a triple quadrupole tandem mass spectrometer, by monitoring the collision-activated decomposition pathway leading to loss of the amide-linked residue of glutamic acid from the protonated molecules. Quantification was performed by isotope dilution, employing as internal standards the custom-prepared analogues of both analytes labelled with a $-CD_3$ group at N^{10}.

The other method is aimed at measuring MTX in environmental samples of air and surface-settled particulates in oncological hospital pharmacies [63] and in the urine of exposed pharmacy technicians [62]. In both cases, MTX was concentrated from the matrix (washing buffer for the environmental samples or urine) by SPE and injected on to a conventional (4 mm I.D.) C_{18} column, which necessitated 1:20 flow splitting to the electrospray ion source. In both cases, OHM was employed as the internal standard for quantification, since this compound is more hydrophobic than MTX and can be eluted more closely to MTX than the much earlier-eluting AMP. A comparison of the sensitivity figures obtained on environmental samples between UV and tandem mass spectrometric detection shows a 30-fold increase obtained with MS–MS.

To date, a single publication reports the application of HPLC–MS with electrospray ionization to the analysis of four monoglutamate folates (FA, FH_4, $10MFH_4$, $5FFH_4$; Fig. 2). Due to lack of strongly basic functions in the folates, compared to MTX, the monitoring of $[M-H]^-$ deprotonated molecules was found to be preferable to positive ions [85].

Gas chromatography–mass spectrometry (GC–MS) has also been employed to measure blood folates in human subjects and in metabolic studies of folic acid as a nutrient which employ stable-isotope labelled FA. The employed analytical approach is very complex and entails extraction of folates from the biological matrix (blood or urine), their chemical degradation by cleavage of the 9c–10N bond and conversion into volatile derivatives amenable to GC–MS. Among the employed derivatives of PABA are: the *N*-trifluoroacetyl-*p*-aminobenzoyl-glutamate lactam-α-trifluoroethylester [86], the *N*,*O*-bis-*t*-butyl-dimethylsilyl derivative of *N*-trifluoroacetyl-

PABA [87] and the *N*-*t*-butyl-dimethylsilyl derivative of *N*-trifluoroacetyl-PABA-ethyl ester [88].

2.4. Analytical performance

Since measurements of drug concentration in biological samples is employed in metabolic, pharmacokinetic and clinical studies often within regulated environments, it is then necessary to perform a method validation, i.e. to provide evidence on the applicability of a proposed analytical method to its stated purpose [89]. Reporting this information is now a stringent requisite to publication of validated bioanalytical assays in *Journal of Chromatography B* [90].

Data on the performance of the considered analytical methods (analyte recovery, the concentration range of the calibration curve, the limits of detection or quantitation, the figures-of-merit for accuracy and precision of sample measurements) reported in Tables 1–3 are taken from the considered papers and converted to homogeneous units. However, especially in the past, full method validation was the exception, rather than the rule, and this explains the heterogeneity (and often the lack) of information on the analytical quality in several papers.

2.4.1. Recovery

The recovery from biological samples of MTX and of its metabolites OHM and DAMPA varies according to the different sample processing techniques (protein precipitation, liquid–liquid extraction, solid-phase extraction) and among the different sample matrices (plasma, serum, urine). It should be remembered, however, that constant, rather than quantitative recovery of the analyte/s is important to obtain a robust assay.

Simple plasma or serum protein precipitation affords a recovery of MTX of 40–96% (11 assays) and of 70–98% for OHM (six assays). Solvent clean-up of the supernatant is an effective procedure for MTX, since its recovery is not reduced (63–96% in seven assays), while for OHM two assays report a recovery around 50%.

The results reported for liquid–liquid extraction of deproteinized samples show that this is not a very convenient procedure, since recovery is generally lower than with the other techniques, due to the intrinsically low solubility of the analytes in the organic solvents. Recovery of MTX by extraction with ethyl acetate/iso-propanol from acid-deproteinized plasma or serum samples is of 38–48% (three assays) and even worse (26%) for OHM. An occasional case of quantitative recovery (96% for MTX, 95% for OHM and 92% for DAMPA) is obtained precipitating serum protein with trichloroacetic acid in ethanol solution and extracting the supernatant with pure ethyl acetate [71].

SPE usually affords high recovery, between 70 and 100% for both MTX (21 assays) and OHM (10 assays), and with an individual case of recovery of MTX below 50% [47]. The occasional data on the other analytes also report comparable figures. It is also noticeable that SPE allows to concentrate trace MTX from large volumes of environmental water samples.

From the available data — and considering that only half of the 72 considered assays report recovery data for at least one of the analytes — SPE can be considered as a more reliable technique than protein precipitation or liquid–liquid solvent extraction; nevertheless, since this technique requires specialized and expensive equipment and substantially higher manual skill from the operators, the other techniques may prove less demanding. In particular, a robotized preparation step involving acetonitrile protein precipitation and chloroform sample clean-up has been incorporated in a recent assay which employs tandem mass spectrometric detection [58].

Chen and Chiou report that MTX contained in standard solutions at concentrations between 1 and 100 mg/l prepared in pure methanol or in 80% ethanol was adsorbed on the walls of glass laboratory glassware, such as volumetric flasks and syringes, with loss as high as 25% for a 1 mg/l solution prepared in pure methanol. In contrast, MTX dilutions in pure water do not show this behaviour at concentrations down to 1 mg/l) [91].

2.4.2. Detection and quantification limits

There is a great deal of confusion over the terms related to the ability of the assay to measure low analyte concentrations. Several heterogeneous criteria can be employed to define limit of detection (LOD) and limit of quantification (LOQ), thus resulting in a difficult comparability of the figures

reported in different papers. A recent step towards harmonization of the requisites of analytical methods employed in bioavailability, bioequivalence and pharmacokinetic studies has been taken, as reported in a summary report of the conference on ''*Analytical methods validation: bioavailability bioequivalence and pharmacokinetic studies*'' (Washington, DC (USA), 3–5 Dec. 1990) [92] and rules have been issued for the publication of validated chromatographic bioanalytical methods in the *Journal of Chromatography* [90]. An operative definition of the quantification limit of an assay has been stated as the lowest concentration of the target analyte in a sample which can be measured with an assigned level of accuracy and precision: within ±20% of the nominal concentration, with a coefficient of variation ≤20% [89,92].

The sensitivity needed for pharmacokinetic measurements of blood concentrations of MTX and OHM in treated patients varies according to the therapeutical doses, which are in the range of 6–8 g/m^2 of body surface for high-dose intravenous antineoplastic treatments and of 10 mg/m^2 for low-dose oral anti-asthmatic treatments. The resulting blood concentrations span between 100 μ*M* (50 μg/ml) at the peak of the pharmacokinetic curve for high-dose treatments [77] and 100 n*M* (50 ng/ml) 24 h after the oral administration [49].

The different preparation protocols applied to the biological samples determine the degree of pre-chromatographic sample enrichment and ultimately the lowest amount of the analytes measurable in the samples. The lower detection limit attained for the measurement of MTX and OHM with ultraviolet detection is in the range between 2 and 20 n*M* in plasma samples processed by SPE, whereas in samples prepared by protein precipitation it is in the 20–200 n*M* range. Assays employing post-column photo-oxidation and fluorescence detection of the degradation products allow lower limits for detection of MTX of 20 n*M* for the direct injection of plasma, urine or ascite samples [73] and of 0.2–2 n*M* for samples processed by SPE. One method employing electrochemical detection featured a detection limit for MTX of 50 n*M* for plasma samples processed by SPE, which could be improved to 2 n*M* by changing the chromatographic column. Tandem mass spectrometric detection allows measurement of MTX and OHM in plasma and urine with detection limits of 0.1–0.4 nM.

2.4.3. Accuracy and precision of measurements

At least partial quantitative data on accuracy and precision of the described analytical methods are reported in most of the papers considered although complete method validation is reported only in one case [58].

Precision is the most often reported parameter, since 49/67 papers report for at least one of the analytes the percent coefficient of variation of repeated measurements of the calibration solutions or, seldom, of quality control samples. Values are in the 10% range for the lower concentrations and less than 2% at the upper end of the calibration curve.

Accuracy is reported in 19/67 papers only, as the relative difference between the measured and target or nominal concentration value of the calibration solutions or of quality control samples. Values are in the ±10% range for the lower concentrations and can be as low as less than ±1% at the upper end of the calibration curve.

The reported values for accuracy and precision fall within the limits of ±15% for the relative difference between the measured and target concentration value of quality control samples and of ±15% for the percent coefficient of variation of repeated measurements of the quality control samples defined in the summary report [92].

2.4.4. Use of an internal standard

Although the use of an internal standard is often considered advantageous in enhancing the quantitative performance of analytical methods, especially when sample preparation includes steps such as liquid–liquid extraction, off-line solid-phase extraction or derivatization, most of the published methods for the quantification of MTX and its metabolites do not use one.

Among the 25/72 assay methods which employ an internal standard, nine use structurally unrelated compounds, such as theophylline [24,71], 8-chlorotheophylline [40,47,55] or 4-amino-acetophenone (*p*AAP) [20,21,23,37].

One of the MTX analogues employed as internal standard is the commercially available aminopterin (*N*-10-*des*-methyl MTX; AMP; Fig. 3.1) [27,28,45,

49,60,70,75]. One problem encountered with AMP is that, under RP conditions, it elutes much faster than MTX, close to the chromatographic front ($0.22<\alpha<0.69$). To avoid obscuring the peak of the internal standard by the fast-eluting sample components, the eluent strength has to be lowered, and thus the elution of MTX is delayed.

To overcome this difficulty, yet employing a close structural analogue of MTX, the metabolite OHM has been employed as the internal standard, since it elutes, under reversed-phase conditions, only slightly later than the target compound [63,62]. This choice is of course justified in the case of the environmental samples, where the metabolite is not expected to occur, but its use may be questioned in the case of the urine analysis. In fact, OHM occurs in the urine of patients treated with high doses of MTX, although at much lower concentration than the parent drug, and its low-level presence has also been sporadically observed, although not confirmed with mass spectrometry, in the urine of exposed nurses [30]. Therefore, the use of OHM as the internal standard in this case may lead to an underestimation of the results.

In a few cases [35,38,39], a custom-synthesized analogue (AQA; Fig. 3.2) is employed, which carries an aspartyl residue in place of the glutamic amino acid in the structure of MTX and elutes before MTX ($0.51<\alpha<0.57$) under reversed-phase conditions, but later ($\alpha=1.87$) under anion-exchange conditions [31].

A single assay [68] employs 8-bromo-methotrexate, which elutes much later than MTX ($\alpha=3.8$).

A single assay [58] employs isotope dilution mass spectrometry to measure MTX and OHM, with the aid of custom synthesized internal standards, labelled with $-CD_3$ at the N^{10} methyl group.

2.4.5. Reference methods and comparison of measurement techniques

Due to the great interest in the therapeutic monitoring of MTX concentration, routine measurement by clinical biochemistry techniques are available in many hospital laboratories on a nearly routine basis. The most commonly employed non-chromatographic analytical techniques are currently the antibody-based fluorescence polarization immunoassay (FPIA, Abbott Laboratories) and the enzyme-multiplied immunoassay (EMIT, Behring Diagnostics). Other techniques include the dihydrofolate-reductase-inhibition assay (DFRInA), the competitive protein binding assay (CPBA), the radio-immunoassay (RIA) and the simple radioassay (RA). One potential disadvantage of the antibody- or protein binding-based techniques is however the low discrimination of molecular recognition between MTX and its metabolites (cross-reactivity), which results in an overestimation of the concentration of unmodified MTX, especially towards the end of the plasma clearance curve. In particular, while the cross-reactivity of the currently employed FPIA and EMIT MTX antibodies towards OHM is very low (0.6 and 4%, respectively), that towards DAMPA is much higher, 83% for the FPIA polyclonal antibody mixture, 41% for the monoclonal FPIA antibody and 100% for the EMIT antibodies [93].

Since HPLC is an analytical technique with very high molecular specificity, at least for small molecules such as drugs and metabolites, it is employed as the gold standard technique to assess the specificity and accuracy of the measurements obtained with other analytical techniques. Comparison between the results of MTX plasma concentrations measured with the different clinical chemistry techniques and those measured by HPLC has been reported in several papers [18,20,33,35,40,41,44,47,52,54,61,64,67,78,94,95]. In most papers, the statistical technique employed to compare the results is a simple linear regression of the concentrations, the ''reference'' technique being the independent variable. In some papers the HPLC results are considered as the independent variable [35,41,95], while in others the ''reference'' analytical technique is the biochemical one [40,47,52,67].

In this simple approach to method comparison, high percentage values of the r^2 correlation coefficient, a value of the slope of the regression line close to unity and a value of the line intercept not statistically different from zero are often taken as ''proofs'' of a good agreement between the two compared techniques. An erroneously considered refinement of this simple evaluation is that proposed by regression of log-transformed results, although this is considered by the authors a technique to reduce the scatter of the data upon visual inspection [52].

Eksborg has strongly questioned the validity of such comparisons, [96], and has thus proposed a different approach, whereby the (alternative method)/HPLC concentration ratio is plotted against the value of MTX concentration measured by HPLC, considered as the reference method [54]. This technique for method comparison has the advantage of immediately showing the precision and accuracy of the method under evaluation throughout the concentration range [61].

2.5. Measurement of MTX levels and calculation of pharmacokinetic parameters

In several papers, the concentration versus time series measured in patients is employed to calculate the pharmacokinetic parameters of the drug and of its main metabolites.

Eksborg [94] measured with different analytical techniques (HPLC, EIA, two different FPIAs and EMIT) the plasma concentration of MTX in 40 individual patients and calculated for each subject the pharmacokinetic parameters, i.e. AUC and $t_{1/2}$, from the MTX concentration versus time data measured with the different techniques. Taking as reference the pharmacokinetic parameters calculated on the basis of the HPLC-measured concentrations, the AUC calculated with the trapezoidal method on the basis of the non-chromatographically measured concentrations resulted between 90 and 150% (25th–75th percentile) and around 40% of the values of the parameter fell outside the 25th–75th percentile range. The median value of $t_{1/2}$ calculated from the data obtained by nonchromatographic methods resulted in a 10% underestimation with one FPIA method, while with the other FPIA method the median value was very close to the HPLC reference; with both FPIA kits 50% of the values fell within the 25th–75th percentile range. The median value of $t_{1/2}$ calculated from the EMIT data was 15% overestimated and only 30% of the calculated values fell within the 25th–75th percentile range. The authors comment that the precision of the analytical method employed to measure the plasma concentration of MTX might substantially influence the subsequent pharmacokinetic evaluation, to the point that misleading conclusions could be drawn on the treatments required by the patient.

2.6. Simultaneous measurement of MTX and folates

The introduction of the folinic acid rescue protocol in high-dose MTX chemotherapy and of the combined 5-fluorouracil–leucovorin protocol prompted for the development of analytical methods for the measurement of the administered folates and of their products of biotransformation. The difference in polarity between MTX and reduced folates is large, so that the separation is quite demanding, especially when it includes multiple folate forms, such as leucovorin and its main metabolite, 5-formyl-tetrahydrofolic acid, along with the metabolites of MTX.

One analytical method employs isocratic elution from a reversed-phase column to resolve a five-component mixture containing leucovorin, N^{10}-MeFH4, MTX, OHM and DAMPA in a 30-min run [78], while other assays resort to gradient elution [77,79], which affords a better resolution within the same analysis time.

A few examples of chromatographic separation of complex mixtures of structural analogues, including monoglutamate MTX and folacins have been reported in the literature (e.g. Refs. [97–100]).

3. Chromatographic assay of “classical” and “non-classical” antitumor antifolates and of their metabolites

3.1. Methods for the analysis of “classical” methotrexate-analog drugs

Some structural analogues of MTX: raltitraxed, lometrexol, multi-targeted antifolate and edatrexate (for their structures, see Fig. 3) are currently being investigated as candidate antitumor drugs, and one (raltitraxed) has been recently licensed for therapeutic use. For the measurement of these drugs and of their metabolites in biological samples, a few analytical methods have been published in the literature. The studies on the poly-glutamyl metabolites of raltitraxed and of the deazapterines are reported in Section 4, together with those regarding the corresponding derivatives of folic acids and methotrexate.

3.1.1. Raltitraxed (Tomudex, ICI D1694, ZD1694)

The structure of raltitraxed (Fig. 3.3) is highly modified with reference to folic acid, since it carries a more hydrophobic substituted quinazoline system in the place of the pterine portion and a 4-amino-thiophene-1-carboxylic acid in the place of the PABA portion. The measurement of the parent drug and of its poly-glutamate metabolites in biological samples has often been accomplished with immunological [101,102], rather than chromatographic techniques. A liquid chromatographic assay has been described for the measurement in plasma, bile and urine of experimental mice and rats of raltitraxed and CB3717, the latter a drug compound the clinical development of which was discontinued due to unacceptable nephrotoxicity [103]. Due to the high hydrophobicity of the analytes, plasma samples were pre-purified by SPE on an octadecyl-silica cartridge, with almost complete recovery. The analytes were eluted isocratically from a hexyl-silica analytical column, which is less retentive than the conventional octadecyl silica towards very lipophilic compounds. Ultraviolet detection (313 nm) allowed the measurement of the analytes at plasma concentrations down to 10 μ*M*. In the more sensitive assays, a structural analogue of raltitraxed, ICI 212281, was employed as the internal standard.

3.1.2. Lometrexol

In the structure of lometrexol (Fig. 3.4), the 2-amino-4-oxo-8-aza-quinazoline portion is linked to the benzoyl-glutamic portion through a methylene bridge. For the determination of lometrexol in biological samples, a variety of analytical approaches have been described similar to those employed for MTX.

Lometrexol was extracted from rat plasma by SPE (recovery of approx. 58%) and measured by employing ion-pair chromatography with hexanesulfonic acid as the pairing reagent on a C_{18}-alkylsilica column with ultraviolet detection. Methotrexate was employed as the internal standard and the minimum detectable concentration was of 10 ng/ml on 0.5-ml samples [104].

In another assay, lometrexol was also extracted from serum by liquid–liquid extraction and the protein-free drug fraction was oxidized by MnO_2 to a fluorescent derivative, which could be separated by gradient elution on a octadecyl-silica column and detected by fluorescence, giving a 5 n*M* lower limit of quantification in human serum and urine [105].

An array of chromatographic methods was developed for the measurement of lomexetrol in human plasma and urine with ultraviolet or fluorimetric detection. All assays use sample pre-purification by SPE and a close structural analogue, C10-*des*-methylene lomexetrol, as the internal standard. The methods employing ultraviolet detection at 288 nm have quantitation limits of 0.2 μg/ml for plasma and of 2 μg/ml for urine and are linear up to concentrations of 10 and 25 μg/ml, respectively. The assay using fluorimetric detection employs preliminary MnO_2 oxidation of the analyte, is 20-fold more sensitive, with a quantitation limit of 0.01 μg/ml) and is linear up to 0.25 μg/ml [106].

An alternative detection technique for the high-sensitivity measurement of lometrexol in plasma employs electrochemical detection. Following sample clean-up, separation is achieved on a phenyl column under isocratic conditions. The calibration curve in plasma is linear from 0.01 to 0.21 μg/ml with inter- and intra-day precision of 5.4 and 5.5%, respectively. The recovery of lometrexol from plasma is 81%, and the lower limit of detection is 5 ng/ml. The assay has comparable sensitivity to fluorimetric methods but does not require the pre-chromatographic oxidation step [107].

3.1.3. Multi-targeted antifolate (MTA; LY231514)

In the molecule of MTA (structure 3.5), the heterocyclic portion is a 2-amino-4-oxo-pyrrolo-pyrimidine, linked to the benzoyl-glutamic portion through a methylene bridge.

To measure the levels of MTA in human plasma, a sensitive method was developed, which involved SPE purification of the samples and injection into a column-switching HPLC system consisting of two YMC-Basic columns. On the pre-column, the analyte and its internal standard are separated from the interferents and then transferred on to the analytical column. Under the assay conditions, the limit of quantitation is 10 ng/ml) and the method is linear from 0.01 to 80 μg/ml [108].

To study the pharmacokinetics and distribution of MTA following intraperitoneal administration, a rat model has been employed and the drug concentration

was measured in the biological fluids (plasma, peritoneal fluid and urine) and in tissues. Preparation of plasma and tissue samples involved extraction of the analyte and of the internal standard, OHM into chloroform, while peritoneal fluid and urine were injected after dilution into the mobile phase. Separation was accomplished by reversed-phase chromatography on a C_{18} column, isocratically eluted at pH 6.5 and the analytes were detected by UV monitoring [109].

3.1.4. Edatrexate and lower homologues

The structure of edatrexate and its homologues (Fig. 3: **6a-c**) is similar to that of methotrexate, with reference to the pterine system and to the presence of the glutamic acid residue, but the N^{10}-methyl function of the PABA system is substituted by a $-CH_2-$ group (10-deaza-aminopterin; 10DAAM; Fig. 3: **6a**) or by a tertiary carbon atom carrying a methyl (10-methyl-10-deaza-aminopterin; 10MDAAM; Fig. 3: **6b**) or an ethyl group (10EdAM; 10-EDAAM; EDAM; Fig. 3: **6c**). The 10-alkyl substituted molecules thus feature a chiral center at C^{10} (structure 3.1). While no chromatographic separation of the C^{10} enantiomers has been reported, the two compounds were separately synthesized and their pharmacological properties compared in vitro on the L210 tumor line and in folate-dependent bacteria and in vivo in the rat, showing no difference in activity, besides a lower acute toxicity of the D-isomer of EDAM [110]. The presence of the metabolites 7-hydroxy-10-EDAM, and 10-ethyl-10-deaza-2,4-diamino-pteroic acid was demonstrated in the plasma and urine of treated patients [111].

A single chromatographic method was developed to measure 10-dAM, 10-EdAM, their polyglutamate anabolites, and their 7-hydroxy (7-OH) and deglutamate catabolites. Plasma, urine and denatured cell culture samples were extracted by SPE on C_{18} sorbent with recoveries of 95% for plasma and of 98% for urine. Analyte separation was accomplished by gradient elution (acetonitrile in 50 m*M* phosphate, pH 7.0) from a reversed-phase C_{18} column and detection by measurement of the high native fluorescence of pteridine-containing compounds which contain carbon in the 10 position. The linear range of the assay is 2–100 n*M* for 10-dAM and 1–100 n*M* for 10-EdAM [112].

To overcome the difficulties inherent in the gradient assay, an isocratic method to measure edatrexate, its 7-hydroxy metabolite, the corresponding pteroic acids and the di- and triglutamate intracellular metabolites was developed. SPE on C_{18} sorbent was used for sample clean-up and adequate accuracy of measurements was obtained without the use of an internal standard. Elution from an octadecyl-silica column was accomplished with a pH 3 mobile phase containing tetramethyl-ammonium and methanol as the organic modifier. Fluorimetric detection with excitation at 243 nm and emission at 488 nm allowed quantification of small amounts of drug or metabolites (2.0–4.0 n*M*, depending on the compound), while ultraviolet detection at 350 nm allowed the measurement of plasma concentrations of 10-EdAM exceeding 50 n*M* [113].

3.2. Methods for the analysis of ''non-classical'' methotrexate-analog drugs

Some ''non-classical'' DHFR inhibitors are well-established as anti-infective agents, and some are currently tested as candidate antitumor drugs. Due to the lack of the aryl-glutamate moiety, these drugs are much more lipophilic than MTX and other ''classical'' antifolates and can thus enter even tumor cells with impaired classical folate transport or cross the blood–brain barrier. A few analytical methods published in the literature for the measurement of some of these drugs: trimetrexate, nolatrexed and piritrexim (for their structures, see Fig. 4) in biological samples are reported in the following.

3.2.1. Trimetrexate

Trimetrexate (Fig. 4: **1**) is a highly hydrophobic, non-glutamylable analogue of MTX, which carries a substituted quinazoline system in place of the pterine portion, a trimethoxy-aniline group in place of the PABA portion and is devoid of the glutamic acid residue. Due to lack of the glutamic acid residue and to the much less polar nature of the heteroaromatic portion of the molecule, the drug promptly enters even tumor cells with impaired folate and MTX transport mechanism [114].

Due to its low polarity, it is possible to measure trimetrexate in biological samples both by gas chromatography–mass spectrometry (GC–MS) and by different HPLC methods. For the GC–MS determination, the plasma sample was extracted by SPE on

C_{18} cartridges and the contained trimetrexate derivatized to yield the *N,N′*-bis-trimethyl-silyl derivative. The minimum detectable level under selected-ion monitoring was 5-14 n*M* [115].

In a pharmacokinetic and metabolism preclinical study in the monkey, the plasma levels of trimetrexate were measured after dosing with [^{14}C]trimetrexate labelled on the *p*-methoxy group with respect to the aniline function. Plasma samples were processed by SPE extraction, employing trimethoprim as an internal standard. The extracts were separated within 10 min on a C_{18}-alkylsilica column eluted in the isocratic mode with a mixture of acetonitrile (23%) and a pH 4.5 phosphate buffer. A modified mobile phase (at pH 7.5 and containing 12% acetonitrile) was employed to screen for plasma metabolites. Urines were directly injected on to a C_{18} column eluted with a gradient of a pH 5 phosphate buffer and acetonitrile (0–60% in 25 min); eluted fractions were measured for radioactivity by scintillation counting and assayed for dihydrofolate-reductase-inhibiting activity. The radioactive materials were collected and re-chromatographed under the same conditions employed to screen plasma metabolites. A combination of chromatographic separation, radiometric and DHFR-inhibition assay allowed to highlight the presence of some unknown metabolites [116].

The stability of trimetrexate in infusion solutions for implanted drug delivery systems was measured at 37°C over a period of 56 days, by employing a reversed-phase HPLC method developed on a C_8 alkylsilica column eluted at mildly acidic pH value with scanning UV detection. The assay highlighted the progressive formation of a photo-decomposition product, identified as (2,4-diamino-5-methyl-6-carboxyaldehyde) by gas chromatography–mass spectrometric analysis and formation of a dinitrophenylhydrazone [117].

The presence of a highly electro-active trimethoxyanilino group in the molecule of trimetrexate allows to use electrochemical detection for the high-sensitivity measurement of the drug and of its metabolites in human plasma, urine and stool samples. In the frame of a phase I study, extracts of biological samples pre-purified by SPE were separated on a C_{18} column eluted isocratically at moderately acidic pH and detected amperometrically at an oxidation potential of 0.6 V. Under these conditions, the lower limit of quantitation in plasma and urine was 50 n*M* and in 20 n*M* in stool [118].

3.2.2. Nolatrexed (AG337; THYMITAQ®)

Nolatrexed (3,4-dihydro-2-amino-6-methyl-4-oxo-5-(4-pyridylthio)-quinazolone; Fig. 4: **2**) is a lipophilic non-glutamylable folate inhibitor featuring a substituted quinazoline ring with a folate substitution pattern. Thymitaq in plasma was assayed by a validated isocratic reversed-phase HPLC assay with detection at 273 nm [119].

3.2.3. Piritrexim (BW 310U; PTX)

Piritrexim (2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-*d*]pyrimidine; Fig. 4: **3**) is a moderately lipophilic ‘‘non-classic’’ antifolate drug. Although a number of metabolic and clinical pharmacokinetics studies are reported in the literature, only two assay methods have been described to measure PTX in plasma samples.

Alkalinized plasma samples containing 40–1000 ng/ml of PTX were extracted with methylene chloride and further purified by SPE on a silica cartridge. The recovery of the analyte was between 80 and 85%. Due to the much higher lipophilicity of PTX with respect to ‘‘classical’’ antifolates, a low-retention C_1 reversed-phase column and a water–acetonitrile (65:35) mobile phase containing octanesulphonate as the ion-pairing reagent were employed to elute the analyte, which was detected by UV absorbance measurement at 254 nm. The lower limit of detection of the assay was approx. 5 ng/ml and the coefficients of variation for the measurement of drug concentrations over the range of 40–1000 ng/ml of plasma was 5% [120]. The same paper also describes an alternative assay by thin-layer chromatography on silica gel plates which allows measurement of amounts of PTX between 20 and 80 ng by scanning densitometry. The lower limit of detection of the assay for plasma samples was approx. 4 ng/ml and the recovery was 89–95%.

4. Application of chromatographic methods to special needs

4.1. Purity analysis of MTX in pharmaceutical preparations

The purity determination of pharmaceutical prepa-

rations is required by regulatory authorities and mandates for assays with a high resolving performance, to separate the largest number of possible contaminants (reaction by-products and decomposition products) present in the main drug, rather than for a very high sensitivity of the assay.

The United States Pharmacopeia (USP, XXI Rev, 1985) contains analytical monographs for the purity determination of folic acid [121a], leucovorin (calcium folinate) [121b] and methotrexate [121c]. In the cases of folic acid and methotrexate, separation is performed on a 25- to 30 cm-long HPLC column packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles, 5–10 μm in diameter [121d] and different elution conditions employed for the analysis of the two drugs. Folic acid is separated with a mixture of methanol and neutral phosphate buffer (pH 7.2), and the resolution requested between calcium formyl-tetrahydrofolate and folic acid is not less than 3.6. Methotrexate is separated with a mixture of acetonitrile and a pH 6 buffer made of dibasic sodium phosphate (Na_2HPO_4) and citric acid, and the resolution requested between methotrexate and folic acid is not less than 8.0. Detection is performed by ultraviolet absorption at 254 nm for folic acid and at 302 nm for methotrexate.

The presence of several byproducts in methotrexate preparations for clinical use has been highlighted in a number of early purity studies, performed by paper electrophoresis [6], DEAE-cellulose column chromatography [7] and paper chromatography [7].

Tong et al. reported the presence of four trace contaminants in methotrexate, by performing reversed-phase separation of the sample at two different values of pH: pH 5 and 3.5 [16]. Under mildly acidic conditions, four contaminants of methotrexate were separated in a 50-min isocratic run, two of which were identified as AMP and DAMPA. Their identity with authentic standards was checked by performing a confirmative separation at a lower value of pH, but elution under these conditions did not improve the separation, although the elution of all compounds was delayed. A much more detailed separation of MTX and its contaminants was obtained later on the same column, by ion-pair chromatography at neutral pH [31]. The analytical conditions employed allowed resolution of 32 peaks, only three of which could be assigned to contaminants of MTX: DAMPA, AMP and methopterin.

A further important facet in the assessment of the chemical purity of drugs with chiral motifs, such as MTX, is the measurement of their enantiomeric purity, which is described below.

4.2. Stereoisomeric analysis of methotrexate and natural folates

The stereoisomeric analysis of methotrexate and of natural folates may be employed to solve two main problems in the pharmacological and therapeutical field.

Some commercial sources of methotrexate were suspected to contain, as a contaminant, the stereoisomer D-MTX, which could arise from racemization of the synthetic product either as a consequence of the reaction conditions adopted for its synthesis (condensation of activated DAMPA with protected glutamic acid) or during the shelf-life of the pharmaceutical product, due to improper storage conditions. The enantiomeric purity of MTX was assessed by chiral HPLC on a RP-C_{18} column by employing a mobile phase containing L-proline and cupric nitrate, and afforded baseline resolution of the two enantiomers in approx. 30 min. The analysis of commercial MTX from several suppliers highlighted that pharmaceutical-grade products had a content of D-MTX between 0.5 and 7%, while a MTX supplied as a chemical standard and not intended for drug use had a content of D-MTX of 48%, thus corresponding to a nearly racemic mixture. Moreover, the availability of an analytical method for the determination of the enantiomers of MTX allowed to follow their different metabolic fate: D-MTX is a good inhibitor of DHFR, but a poor inhibitor of cell growth and is not hydrolyzed to DAMPA by carboxypeptidase G_1, a prospective rescue agent. The rate of elimination of both MTX enantiomers from plasma of dosed mice appears to be essentially the same [122].

Leucovorin (5-CHO-THFA; folinic acid) is a reduced folate which has long been used as a rescue agent in intermediate and high-dose MTX chemotherapy and in a combined 5-fluorouracil/leucovorin chemotherapy association in vivo, leucovorin is converted to the metabolite 5-methyl-tetrahydrofolate. Although the molecules of folinic acid and of

5-methyl-tetrahydrofolate have two chiral centers: one at C_6 of the pterine ring, the other on the unit of glutamic acid, the natural products contain only D-glutamic acid. The commercially available leucovorin is a 1:1 mixture of [6*R*]- and [6*S*]-isomers, the latter being known as citrovorum factor. Since it has been found that the [6*R*]-isomer of folinic acid has a considerably longer half-life in plasma than the [6*S*]-isomer, the separate measurement of the two stereoisomers of 5-formyl-tetrahydrofolate and of 5-methyl-tetrahydrofolate by chromatography is an appealing alternative to the traditional bio-assay based on the growth of folate-dependent microorganisms, which may be interfered by cross-reacting or inhibiting compounds, and therefore some methods have been published on the measurement of folinic acid and of its metabolite by HPLC [123–125].

Quantifying the two enantiomers of leucovorin and of its metabolite 5-Me-THFA in patients, who may be contemporarily treated with MTX, is indeed a difficult analytical problem. The separation of folate diastereoisomers can be achieved on a chiral column composed of bovine serum albumin immobilized on silica and eluted isocratically with a neutral phosphate buffer [126,127]. When it is needed to pre-separate the individual pterine compounds in the sample (e.g. leucovorin from MTX), before performing chiral chromatography, this can be accomplished on-line, on a C_8-phenyl or on a conventional C_{18} apolar column; the peak corresponding to the desired component is then loaded on the chiral column by direct column switching [128,129].

4.3. Environmental and biological monitoring of MTX in occupational hygiene

It has long been known that some workers, among whom are those in the pharmaceutical industry, as well as hospital pharmacy technicians and oncologic nurses, can be occupationally exposed to traces of antineoplastic drugs. Since some drugs, such as cyclophosphamide, are known human carcinogens, their manipulation may pose a health risk to workers [8]. MTX is not a carcinogen, but is known to be an experimental teratogen and has increasingly wide clinical applications; not only in oncologic chemotherapy, but also as an immunosuppressive agent. Thence stems the necessity to monitor both the level of workplace contamination and the presence of MTX in the urine of potentially exposed workers. The performances of the analytical methods are peculiar: analytical methods employed in workplace monitoring for industrial hygiene purposes need to be simple and relatively inexpensive. Methods for biological monitoring need a very high sensitivity, since the doses of the drugs to which workers are potentially exposed are orders of magnitude lower than therapeutic ones and virtually absolute specificity, since the number of false positives must be kept at a minimum.

Airborne MTX in the atmosphere of pharmaceutical compounding facilities can be collected on glass fiber filters without degradation, even after prolonged storage of the collection devices [130]. The dust trapped on the filter is dissolved in an aqueous neutral buffer and MTX is chromatographically separated and detected under conditions close to those of the USP, which allowed detection of airborne traces of MTX of approx. 0.3 $\mu g/m^3$ dispersed in the indoor air in the pharmacy of hospital oncological departments during the preparation of patients' doses [131].

The same technique has been applied to the measurement of the contamination of surfaces from traces of MTX in drug formulation plants and in hospital pharmacies. The contamination on surfaces can be collected by ''cleaning'' them with tissues (''swabs'') moistened with or dipped into suitable water-based solutions (either neutral or mildly acidic), which are then analyzed to detect and measure the target contaminant. The analytical sensitivity of the methods allowed measurement of contamination in the range of 0.02–6 $\mu g/m^2$ on the tray of the preparation hood and levels of 20–1900 μg on pairs of gloves donned by pharmacy technicians [132,133].

Earlier analytical conditions employed in these studies, however, suffer from the excessive duration of the analysis, which is inconvenient in the field of occupational hygiene, since numbers of samples have to be analyzed. More recently, a sensitive analytical method entailing a much shorter analysis time under isocratic conditions has been published [28]. A short analytical column packed with 3 μm

ODS material and elution with a pH 6 buffer containing 4% acetonitrile yielded sufficient resolving power to measure MTX by direct injection of surface washing samples at levels as low as 50 ng/ml, a limit corresponding to detection of contamination in the range between 2 and 20 $\mu g/m^2$, depending on the extension of wiped surface (0.05–0.5 m^2). Moreover, LoD can be easily enhanced to 0.02–1 $\mu g/m^2$, since solutions can be easily concentrated 20- to 100-fold by SPE on conventional 100-mg ODS cartridges. Although MTX is an antineoplastic drug employed in relatively low amounts with respect to other drugs, such as cyclophosphamide and 5-fluorouracil, MTX has to be considered a promising tracer of drug indoor contamination, since it is easy and cheap to measure routinely.

Biological monitoring of workers' exposure to MTX was performed on urine samples of hospital nurses caring for osteosarcoma patients treated with high doses of MTX. Nurses who prepared a total amount of 20 g of MTX excreted a cumulative amount of 0.05–1.4 mg of the drug, while in the urine of the nurses who tended the patients the cumulative amount of MTX excreted was of 6–19 μg [30,134].

4.4. Chromatographic determination of polyglutamyl folates and related drugs

Although methotrexate has been in clinical use for several decades, various facets of its mechanism of action are still a subject for fundamental biochemical and pharmacological research both in vivo and in vitro. The main intracellular biotransformation pathway of natural folates and of ''classical'' antifolate drugs is the sequential addition of γ-linked units of glutamic acid. Since these compounds are postulated to be the true ligands of some enzymes in the folate utilization pathways, a research subject of great importance in the design of novel antifolate drugs is the measurement of the individual γ-glutamylated oligomers of natural folates and of their drug counterparts carrying modified pterine rings, in tissue homogenates and cell cultures. Experiments are performed in cell cultures, in animal models and in human subjects or patients and require specific and sensitive analytical methods.

Earlier work relied on a specific reductive cleavage reaction taking place at the bond between C^9 and N^{10} and leading to the *p*-aminobenzoyl-polyglutamates, which were isolated by charcoal adsorption and characterized by anion-exchange chromatography on a DEAE-cellulose column [135,136]; however, when this approach is applied to natural mixtures, the specification of the individual pterine portions of the different molecular species is lost.

More recently, complementary chromatographic approaches have been employed to separate the intact γ-glutamyl oligomers of the natural folates and of several drug analogues (the folacins FA, FH2, FH4, 5MFH4, 10FFH4, MTX and its metabolite OHM, raltitraxed, 10-deaza-aminopterin, 10-deaza-10-ethyl-aminopterin and its 7-hydroxy metabolite) in tissue samples: gel permeation chromatography, anion-exchange liquid chromatography and conventional or ion-pair reversed-phase chromatography. When the concentration of analytes was sufficiently high, detection was accomplished by spectrophotometry; in a large number of in vitro experiments, radioactive-labelled drugs were incubated and detection of polyglutamates present in minute amounts was obtained by off-line scintillation counting of the eluted fractions or detection. In some cases, an independent characterization of the identity of the eluted compounds was performed by hydrolyzing the polyglutamates with pteroyl-polyglutamate hydrolase from fresh human serum and identifying the released MTX on the same chromatographic system. As will be described below, the identification of the number of glutamyl residues in an individual oligomer can be obtained even in the absence of authentic standards, due to their regular elution pattern under various chromatographic conditions. In a few cases, polyglutamates of synthetic origin or extracted from biological samples were also characterized by mass spectrometric techniques.

Gel permeation chromatography on a Sephadex G-15 column eluted with a neutral phosphate buffer was employed to measure MTX polyglutamates in the supernatant of deproteinized tissue homogenates of experimental animals treated with a very low amount (40 μg/kg per day) of [^{3}H]MTX [137,138]. As expected, the individual γ-glutamyl oligomers elute in the order of their decreasing molecular mass, i.e. with MTX eluting last.

Separation by ion-exchange of folates and drug analogues was performed either at low pressure, on a DEAE-Sephadex weak anion-exchange column [139–141], or on a SAX strong anion-exchange HPLC column [142,143]. Elution from both columns was accomplished with a NaCl gradient, at neutral pH on the DEAE-Sephadex column, and at pH 6.5 [143] or 3.3 [142] on the SAX column. As expected, elution occurred starting from MTX, in the increasing order of the number of glutamyl residues, irrespective of the pH value of the eluting buffer.

MTX-polyglutamates as a homogeneous chemical group of substances group were eluted in front of unmodified MTX under reversed-phase conditions with a short isocratic run, by employing an acidic buffer [68].

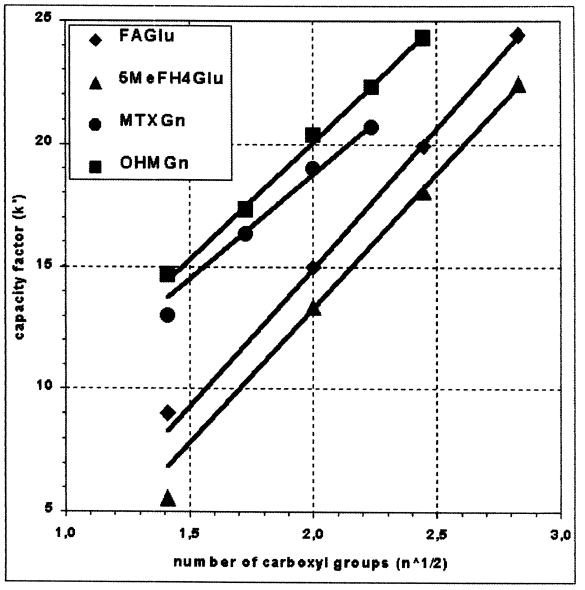
Resolution of the individual γ-glutamyl oligomers on octadecyl-silica reversed-phase columns can be achieved under several chromatographic conditions. In some cases, isocratic elution at pH 2.3 [70] or at pH 4.7 [144] has been employed, however leading to an excessive length of the run in order to resolve the early-eluting highly polyglutamylated oligomers. Therefore, conventional gradient elution is employed to improve the separation. The most frequently described conditions employ gradients between acetonitrile and aqueous buffers at various values of pH: phosphoric acid at pH 2.3 was employed to resolve MFH4 oligomers [145]; acetate buffer at a pH value of 5–5.5 was employed to separate the oligomers of FA [145], MTX [146–149], OHM [149] and DAAP [141]; phosphate at pH 5 [150] or Tris–phosphate at pH 5.5 to separate those of five folates (FA, FH2, FH4, 5MFH4, 10FFH4) [151] and phosphate at pH 7.0 to resolve those of 10EDAAM [112]. When the pH of the eluent is low, ionization of the carboxyl groups is suppressed and the oligomers are eluted in the order of increasing number of glutamyl residues. In contrast, at neutral pH of the eluent, the carboxyl groups are predominantly ionized and the oligomers are eluted in the order of decreasing hydrophobicity, i.e. in decreasing order of the number of γ-glutamyl residues, the latest-eluting being the unmodified drug. This behaviour is very convenient to oligomer identification purposes, since it allows to confirm the identity of individual compounds.

Ion-pairing with cationic [152–154] or anionic [155] reagents was also employed to resolve the oligomers of MTX, OHM and raltitraxed. When ion-pair chromatography is employed with tetrabutyl-ammonium as the pairing reagent at pH 7 and with a gradient of methanol or acetonitrile as the organic modifier, the individual γ-glutamyl oligomers are eluted starting from MTX, in the order of increasing number of glutamyl residues, since the poly-glutamylated oligomers with a higher number of amino acid residues form with the large organic cation increasingly more hydrophobic ion pairs [152,154,156,157]. In contrast, when using hexanesulfonic acid as the pairing reagent at pH 3.75 and a methanol gradient, the oligomers are eluted in the order of increasingly hydrophobic character, i.e. starting from those with longer poly-glutamyl chains [155]. van Tellingen and coworkers report the separation of a complex mixture of compounds related to the investigational drug 10EDAAM, which included the synthetic mono- and di-glutamate metabolites of the parent drug and of the 7-hydroxy metabolite [113]. A linear gradient separation was performed under ion pairing conditions with tetramethyl-ammonium at pH 3. In this case, the more glutamylated drug oligomers elute before the parent compounds, pointing to ineffective ion-pairing at acidic pH.

The separation by ion-exchange or reversed-phase chromatography of the poly-glutamyl oligomers of several pterines (folic acid, N^5-methyl-folic acid, methotrexate and 7-hydroxy-methotrexate) yields nice empirical relationships between the adjusted retention time of each γ-glutamyl oligomer and the number of glutamyl residues. This phenomenon was first observed in the anion-exchange separation of the oligoglutamates of the folic acids by Stout et al. [143]. Since then, several authors have employed such relationships to strengthen the confidence in the identification of the isolated compounds, when authentic reference samples were not easily available and absolute characterization by spectroscopic means has not yet been obtained. Several plots of the experimental results have been employed to calculate line equations between elution and degree of glutamylation, aiming to predict (by interpolation [154] or by extrapolation [158]) the expected retention time of the (usually highly) glutamylated homologues. The employed chromatographic parameter is the capacity factor (k') of each individual γ-glutamyl oligomer, while the structure-related de-

scriptor is related to the number of free carboxyl groups.

As exemplified in Fig. 7, a nice linear relationship can be observed between the degree of glutamylation, expressed as the square root of the number of carboxyl groups in the molecule (i.e. $(n+2)^{0.5}$, n being the number of added γ-glutamyl units to the parent molecule) and the retention factors of some oligomers of MTX [153,154], OHM [153], FA and MeFH4 [143]. It is surprising to notice that, in the cases exemplified in the figure, the lines obtained have very close values of the slope, although the employed separation conditions are very different: in fact, the oligomers of MTX and OHM were separated by gradient elution with tetrabutyl-ammonium reversed-phase ionpairing [153], while the folic acids were separated by strong anion-exchange chromatography [143]. The similarity of the two trends is however less surprising, if it is considered that in both cases the increasing number of carboxyl groups yields a higher retention of the individual oligomer on to the stationary phase.



FAG_n: Y = -7,86 ± 0,46 * 11,40 ± 0,22 * X r^2=0,9996
5-Me-$FH4G_n$: Y = -8,62 ± 0,43 + 10,96 ± 0,20 *X r^2=0,9997
$MTXG_n$: Y = 1,50 ± 1,02 + 8,63 ± 0,59 * X r^2=0,9954
$OHMG_n$: Y = 0,77 ± 0,64 * 9,65 ± 0,34 * X r^2=0,9982

Fig. 7. Structure–retention relationships of the polyglutamates of folic acid ($FAGlu_n$; ♦) and 5-methyl-tetrahydrofolic acid ($MeFH4Glu_n$; ▲), methotrexate ($MTXG_n$; ●); 7-hydroxy-methotrexate ($OHMG_n$; ■), The horizontal axis (X) reports the structural descriptor of the molecules (the square root of the number of carboxyl groups in the molecules). The vertical axis (Y) reports the adjusted retention times (k') of the individual compounds, recalculated from the chromatographic data reported in the references. The data for $MTXG_n$ and $OHMG_n$ are taken from Ref. [149], those for $FOLG_n$ and $MeFH4G_n$ from Ref. [143]. The regression equations are reported below the graph.

However, the above described data transformation is not able to linearize the retention–structure relationship of the oligomers obtained under chromatographic conditions which reverse the elution order, i.e. when the more hydrophilic highly glutamylated oligomers are eluted before the unmodified drug (the most hydrophobic compound). This behaviour is observed, e.g. for MTX under isocratic conditions at acidic pH, without ion-pairing reagents [143,144] and under gradient elution with hexanesulfonate reversed-phase ion-pairing [155], for 10DAAM and 10EDAAM under gradient elution at pH 5.1 [141] and for 10EDAAM and its 7-hydroxy metabolite under isocratic conditions at pH 3 [113]. In these cases, to obtain an equivalent linear relationship as a function of the number of glutamate residues, the reciprocal of the square root of the capacity factor ($k'^{-0.5}$) was plotted against the total number of carboxyl groups in the oligomer, as exemplified in Fig. 8.

Two papers report separations of MTX glutamate oligomers obtained on the same brand of C_{18} column and with a very similar mobile phase, containing tetrabutyl-ammonium as the ion-pairing reagent in a neutral buffer, but eluted with a different organic modifier (methanol in [153] vs. acetonitrile in [154]) and with different gradient shapes. The slope values calculated for the relationship between chromatographic retention and glutamyl chain length of MTXGn oligomers in the two cases are very different, that obtained with methanol being nearly one-third that with acetonitrile.

Selhub reported nice reversed-phase gradient separations of the γ-glutamyl oligomers ($1<n<7$) of different folacins: FA, FH2, FH4, 5*N*-Me-FH4 and 10*N*-HCO-FH4, obtained on a phenyl-silica column by elution at acidic pH [150] or on an octadecyl-silica with tetrabutyl-ammonium as the ion-pairing reagent [151]. Under these conditions, folate glutamyl oligomers eluted in increasing order of the respective number of glutamate residues; however,

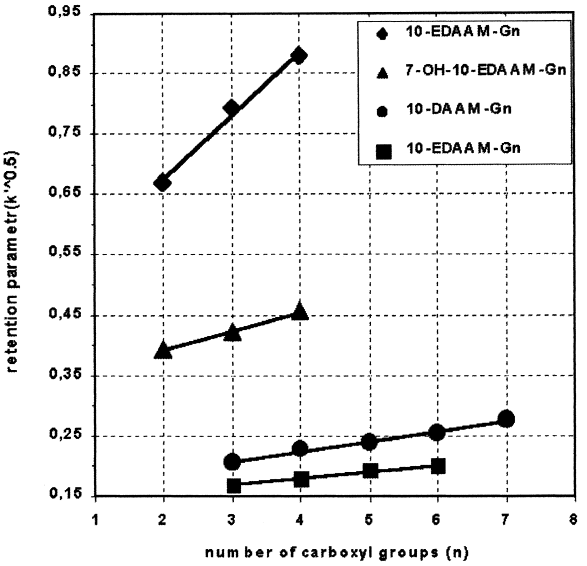


Fig. 8. Structure–retention relationships of the polyglutamates of 10-deaza-aminopterin (10DAAM-G_n ● [141]), 10-ethyl-10-deaza-aminopterin (10EDAAM-G_n; ♦ [113]; ■ [141]) and 7-hydroxy-10-ethyl-10-deaza-aminopterin (7OH10DAAM-G_n; ▲ [113]), separated by gradient reversed-phase chromatography. The horizontal axis (X) reports the structural descriptor of the molecules (the number of carboxyl groups in the molecules). The vertical axis (Y) reports the transformed capacity factors ($k'^{-0.5}$) of the individual oligomers, recalculated from the chromatographic data reported in the cited references.

the plot of their capacity factors as a function of the number of carboxyl groups yields non linear convex trends.

Finally, poly-glutamyl oligomers of folic acids and of drug analogues, either of synthetic origin or extracted from biological samples have been characterized by mass spectrometry with modern soft ionization techniques, such as fast atom bombardment (FAB) [141] and matrix-assisted laser desorption/ionization (MALDI) [159].

5. Conclusions

This review encompasses 25 years of analytical research and practice in the measurement of methotrexate, of structurally analogue drugs, of their metabolites and of related folic acids in chemical, biological, clinical and even environmental research.

The knowledge acquired so far on the chromatographic behaviour of the analytes currently allows to perform sensitive measurements of MTX concentration in treated patients within short times, not only with state-of-the-art yet expensive and highly specialized instrumentation, such as on tandem mass spectrometers coupled to liquid chromatographs, but also on simple and inexpensive chromatographic equipment, which is widely available even in the clinical environment. Availability of fast and specific chromatographic assays with a potential for automated sample preparation makes them excellent competitors to conventional clinical chemistry assays to improve the clinical management of antineoplastic chemotherapy with methotrexate on the basis of accurate measurements of drug and metabolite concentration.

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References

[1] Anon., Methotrexate, in: C. Dollery (Ed.), Therapeutic Drugs, 2nd Edition, Churchill Livingstone, Edinburgh, 1999, M90–96.
[2] D.R. Newell, Semin. Oncol. 26 (2 Suppl. 6) (1999) 74.
[3] D.A. Cairnes, W.E. Evans, Ther. Drug Monit. 5 (3) (1983) 363.
[4] K. Sasaki, R. Hosoya, Y.M. Wang, G.L. Raulston, Biochem. Pharmacol. 32 (3) (1983) 503.
[5] D.M. Valerino, Lancet ii (1972) 1025.
[6] G. Tisman, W.A. Winsten, Lancet i (1970) 1178.
[7] D.M. Valerino, D.G. Johns, D.S. Zaharko, V.T. Oliverio, Biochem. Pharmacol. 21 (1972) 821.
[8] P.J.M. Sessink, R.P. Bos, Drug Saf. 20 (1999) 347.
[9] S. Eksborg, H. Ehrsson, J. Chromatogr. 340 (1985) 31.
[10] M. Fleisher, Ther. Drug Monit. 15 (1993) 521.
[11] A.R. Chamberlin, Anal. Prof. Drug Subst. 5 (1976) 283.

[12] L.O. Pont, Anal. Prof. Drug Subst. 8 (1979) 315.
[13] F.J. Al-Shammary, K.A. Al-Rashood, N.A.A. Mian, M.S. Mian, Anal. Prof. Drug Subst. 19 (1990) 221.
[14] D.C. Chatterji, J.F. Gallelli, J. Pharm. Sci. 66 (1977) 1219.
[15] J. Lankelma, H. Poppe, J. Chromatogr. 149 (1978) 587.
[16] W.P. Tong, J. Rosenberg, D.B. Ludlum, Lancet ii (1975) 719.
[17] T. Okuda, M. Motohashi, I. Aoki, T. Yashiki, J. Chromatogr. B 662 (1) (1994) 79.
[18] J.A. Nelson, B.A. Harris, W.J. Decker, D. Farquhar, Cancer Res. 37 (11) (1977) 3970.
[19] Z. Yu, D. Westerlund, J. Chromatogr. A 742 (1-2) (1996) 113.
[20] T.A. Najjar, K.M. Matar, I.M. Alfawaz, Ther. Drug Monit. 14 (2) (1992) 142.
[21] M. Stout, Y. Ravindranath, R. Kauffmann, J. Chromatogr. 342 (1985) 424.
[22] F. Palmisano, A. Guerrieri, P.G. Zambonin, T.R. Cataldi, Anal. Chem. 61 (9) (1989) 946.
[23] J. Dutrieu, Y.A. Delmotte, Fresenius' Z. Anal. Chem. 315 (1983) 539.
[24] F. Palmisano, T.R. Cataldi, P.G. Zambonin, J. Chromatogr. 344 (1985) 249.
[25] T. Suzuki, H. Hashimoto, N. Ichimin, Fresenius J. Anal. Chem. 351 (1995) 806.
[26] S. Emara, H. Askal, T. Masujima, Biomed. Chromatogr. 12 (6) (1998) 338.
[27] M. Okamoto, I. Yoshida, M. Utsumi, K. Nobuhara, K. Jinno, J. Chromatogr. 515 (1990) 43.
[28] L. Floridia, A.M. Pietropaolo, M. Tavazzani, F.M. Rubino, A. Colombi, J. Chromatogr. B 726 (1-2) (1999) 95.
[29] E. Raude, M. Oellrich, M. Wrenger, Fresenius' Z. Anal. Chem. 330 (1988) 384.
[30] R.M. Mader, B. Rizovski, G.G. Steger, H. Rainer, R. Proprentner, R. Kotz, J. Chromatogr. 613 (2) (1993) 311.
[31] E. Watson, J.L. Cohen, K.K. Chan, Cancer Treat. Rep. 62 (3) (1978) 381.
[32] J.L. Wisnicki, W.P. Tong, D.B. Ludlum, Cancer Treat. Rep. 62 (4) (1978) 529.
[33] C. Canfell, W. Sadee, Clin. Chem. 25 (1979) 1063.
[34] J. Lankelma, E. van der Kleijn, F. Ramaekers, Cancer Lett. 9 (1980) 3970.
[35] R.G. Buice, W.E. Evans, J. Karas, C.A. Nicholas, P. Sidhu, A.B. Straughn, M.C. Meyer, W.R. Crom, Clin. Chem. 26 (13) (1980) 1902.
[36] W.P. Tong, J.L. Wisnicki, J. Horton, D.B. Ludlum, Clin. Chim. Acta 107 (1–2) (1980) 67.
[37] J.L. Cohen, G.H. Hisayasu, A.R. Barrientos, M.S. Nayar, K.K. Chan, J. Chromatogr. 181 (1980) 478.
[38] G.J. Lawson, P.F. Dixon, G.W. Aherne, J. Chromatogr. 223 (1) (1981) 225.
[39] R.G. Buice, P. Sidhu, J. Pharm. Sci. 71 (1981) 74.
[40] C.P. Collier, S.M. MacLeod, S.J. Soldin, Ther. Drug Monit. 4 (4) (1982) 371.
[41] N. So, D.P. Chandra, I.S. Alexander, V.J. Webster, D.W. O'Gorman-Hughes, J. Chromatogr. 331 (1) (1985) 81.
[42] A. el-Yazigi, M. Amer, I. Al-Saleh, C. Martin, Int. J. Cancer 38 (6) (1986) 795.
[43] R. Erttmann, S. Bielack, G. Landbeck, Oncology 43 (2) (1986) 86.
[44] L. Slordal, P.S. Prytz, I. Pettersen, J. Aarbakke, Ther. Drug Monit. 8 (3) (1986) 368.
[45] P.A. Brimmell, D.J. Sams, J. Chromatogr. 413 (1987) 320.
[46] K. van der Steuijt, P. Sonneveld, J. Chromatogr. 422 (1987) 328.
[47] W. Cosolo, O.H. Drummer, N. Christophidis, J. Chromatogr. 494 (1989) 201.
[48] E. Brandsteterova, O. Seresova, S. Miertus, V. Reichelova, Neoplasma 37 (4) (1990) 395.
[49] T.P. Assadullahi, E. Dagli, J.O. Warner, J. Chromatogr. 565 (1–2) (1991) 349.
[50] O. Beck, P. Seidemann, M. Wennberg, C. Peterson, Ther. Drug Monit. 13 (1991) 528.
[51] T. Hirai, M. Kitamura, Y. Inoue, Yakugaku Zasshi 114 (8) (1994) 602.
[52] M. Cociglio, D. Hillaire-Buys, C. Alric, J. Chromatogr. B 674 (1) (1995) 101.
[53] G. Lu, H. Won Jun, J. Liq. Chromatogr. 18 (1995) 155.
[54] F. Albertioni, C. Rask, S. Eksborg, J.H. Poulsen, B. Pettersson, O. Beck, H. Schroeder, C. Peterson, Clin. Chem. 42 (1996) 39.
[55] H. Aboleneen, J. Simpson, D. Backes, J. Chromatogr. B 681 (2) (1996) 317.
[56] Z. Yu, D. Westerlund, K.-S. Boos, J. Chromatogr. B 689 (1997) 379.
[57] T. Hirai, S. Matsumoto, I. Kishi, J. Chromatogr. B 690 (1-2) (1997) 267.
[58] S. Steinborner, J. Henion, Anal. Chem. 71 (13) (1999) 2340.
[59] A. Sparreboom, W.J. Loos, K. Nooter, G. Stoter, J. Verweij, J. Chromatogr. 735 (1999) 111.
[60] E.A. McCrudden, S.E. Tett, J. Chromatogr. B 721 (1) (1999) 87.
[61] H. Seidel, A. Andersen, J.T. Kvaloy, R. Nygaard, P.J. Moe, G. Jacobsen, B. Lindqvist, L. Slordal, Leuk. Res. 24 (3) (2000) 193.
[62] R. Turci, M.L. Fiorentino, C. Softani, C. Minoia, Rapid Commun. Mass Spectrom. 14 (3) (2000) 173.
[63] R. Turci, G. Micoli, C. Minoia, Rapid Commun. Mass Spectrom. 14 (8) (2000) 685.
[64] S.K. Howell, Y.M. Wang, R. Hosoya, W.W. Sutow, Clin. Chem. 26 (6) (1980) 734.
[65] Y.-M. Wang, S.K. Howell, J.A. Benvenuto, J. Liq. Chromatogr. 3 (1980) 1071.
[66] M.L. Chen, W.L. Chiou, J. Chromatogr. 226 (1981) 125.
[67] C. Canfell, W. Sadee, Cancer Treat. Rep. 64 (1) (1980) 165.
[68] B. Nuernberg, M. Kohlbrenner, R. Faulkner, D.E. Furst, J. Chromatogr. 487 (2) (1989) 476.
[69] H. Breithaupt, E. Kuenzler, G. Goebel, Anal. Biochem. 121 (1982) 103.
[70] P. Sonneveld, F.W. Schultz, K. Nooter, K. Hahlen, Cancer Chemother. Pharmacol. 18 (2) (1986) 111.
[71] H.N. Alkaysi, A.M. Garaibeh, M.A. Salem, Ther. Drug Monit. 12 (1990) 191.
[72] F. Albertioni, B. Pettersson, O. Beck, C. Rask, P. Seideman, C. Peterson, J. Chromatogr. B 665 (1) (1995) 163.
[73] J. Salamoun, J. Frantisek, J. Chromatogr. 378 (1) (1986) 173.
[74] J. Salamoun, M. Smrz, F. Kiss, A. Salamounova, J. Chromatogr. 419 (1987) 213.

[75] D.A. Cairnes, W.E. Evans, J. Chromatogr. 231 (1982) 103.
[76] S.M. El-Dareer, K.F. Tillery, D.L. Hill, Cancer Treat. Rep. 65 (1–2) (1981) 101.
[77] S. Belz, C. Frickel, C. Wolfrom, H. Nau, G. Henze, J. Chromatogr. B 661 (1) (1994) 109.
[78] R.C. Donehower, K.R. Hande, J.C. Drake, B.A. Chabner, Clin. Pharmacol. Ther. 26 (1979) 63.
[79] O. van Tellingen, H.R. van der Woude, J.H. Beijnen, C.J.T. van Beers, W.J. Nooyen, J. Chromatogr. 488 (1989) 379.
[80] T. Kiffmeyer, H.J. Gottze, M. Jursch, U. Luders, Fresenius J. Anal. Chem. 361 (1998) 185.
[81] T. Rebello, Anal. Biochem. 166 (1) (1987) 55.
[82] M.C. Kirk, W.C. Coburn Jr., J.R. Piper, Biomed. Mass Spectrom. 3 (5) (1976) 245.
[83] C.E. Hignite, D.L. Azarnoff, Biomed. Mass Spectrom. 5 (2) (1978) 161.
[84] M. Przybylski, J. Preiss J., R. Dennebaum, J. Fischer, Biomed. Mass Spectrom. 9 (1) (1982) 22.
[85] P. Stokes, K. Webb, J. Chromatogr. A 864 (1) (1999) 59.
[86] J.F. Gregory 3rd, J.P. Toth, Anal. Biochem. 170 (1) (1988) 94.
[87] C.R. Santhosh-Kumar, J.C. Deutsch, K.L. Hassell, N.M. Kolhouse, J.F. Kolhouse, Anal. Biochem. 225 (1) (1995) 1.
[88] S.R. Dueker, Y. Lin, A.D. Jones, R. Mercer, E. Fabbro, J.W. Miller, R. Green, A.J. Clifford, Anal. Biochem. 283 (2) (2000) 266.
[89] F. Bressolle, M. Bromet-Petit, M. Audran, J. Chromatogr. B 686 (1996) 3.
[90] W. Lindner, I.W. Wainer, J. Chromatogr. B 707 (1998) 1.
[91] M.L. Chen, W.L. Chiou, J. Pharm. Sci. 71 (1982) 129.
[92] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cok, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309.
[93] B.C. Widemann, F.M. Balis, P.C. Adamson, Clin. Chem. 45 (1999) 223.
[94] S. Eksborg, F. Albertioni, C. Rask, O. Beck, C. Palm, H. Schroeder, C. Peterson, Cancer Lett. 108 (2) (1996) 163.
[95] W. Martz, M. Oellerich, G. Schumann, J. Clin. Chem. Clin. Biochem. 28 (12) (1990) 951.
[96] S. Ekborg, Clin. Chem. 27 (1981) 1312.
[97] D.L. Holt, R.L. Wehling, M.G. Zeece, J. Chromatogr. 449 (1) (1988) 271.
[98] M. Kohashi, K. Inoue, H. Sotobayashi, K. Iwai, J. Chromatogr. 382 (1986) 303.
[99] D.S. Duch, S.W. Bowers, C.A. Nichol, Anal. Biochem. 130 (2) (1983) 385.
[100] A. Hahn, K.H. Flaig, G. Rehner, J. Chromatogr. 545 (1) (1991) 91.
[101] A.L. Jackman, G.A. Taylor, W. Gibson, R. Kimbell, M. Brown, A.H. Calvert, I.R. Judson, L.R. Hughes, Cancer Res. 51 (20) (1991) 5579.
[102] G.W. Aherne, E. Ward, N. Lawrence, D. Dobinson, S.J. Clarke, H. Musgrove, F. Sutcliffe, T. Stephens, A.L. Jackman, Br. J. Cancer 77 (2) (1998) 221.
[103] D.I. Jodrell, D.R. Newell, W. Gibson, L.R. Hughes, A.H. Calvert, Cancer Chemother. Pharmacol. 28 (5) (1991) 331.
[104] O. van Tellingen, J.H. Sips, J.H. Beijnen, J.H. Schornagel, W.J. Nooyen, J. Chromatogr. 576 (1) (1992) 158.
[105] J.R. Muindi, C.W. Young, C. Shih, J. Chromatogr. 621 (1) (1993) 55.
[106] S.R. Wedge, S. Laohavinij, G.A. Taylor, D.R. Newell, J. Chromatogr. B 663 (2) (1995) 327.
[107] T.W. Synold, B. Xi, E.M. Newman, F.M. Muggia, J.H. Doroshow, J. Chromatogr. B 683 (2) (1996) 245.
[108] C.L. Hamilton, J.A. Kirkwood, J. Chromatogr. B 654 (2) (1994) 297.
[109] S.R. Pestieau, O.A. Stuart, P.H. Sugarbaker, Eur. J. Surg. Oncol. 26 (7) (2000) 696.
[110] J.I. DeGraw, P.H. Christie, H. Tagawa, R.L. Kisliuk, Y. Gaumont, F.A. Schmid, F.M. Sirotnak, J. Med. Chem. 29 (6) (1986) 1056.
[111] L. Williams, F. Farag, C.W. Young, Cancer Res. 48 (1988) 5573.
[112] J.J. Kinahan, L.L. Samuels, F. Farag, M.P. Fanucchi, P.M. Vidal, F.M. Sirotnak, C.W. Young, Anal. Biochem. 150 (1985) 203.
[113] O. van Tellingen, J.H. Beijnen, H.R. van der Woude, P.F. Bruning, W.J. Nooyen, J. Chromatogr. 529 (1990) 135.
[114] J.L. Marshall, R.J. DeLap, Clin. Pharmacokinet. 26 (1994) 190.
[115] P.L. Stetson, W.D. Ensminger, J. Chromatogr. 383 (1) (1986) 69.
[116] F.M. Balis, C.M. Lester, D.G. Poplack, Cancer Res. 46 (1) (1986) 169.
[117] P.L. Stetson, U.A. Shukla, W.D. Ensminger, J. Chromatogr. 464 (1) (1989) 163.
[118] J.T. Lin, A.R. Cashmore, M. Baker, R.N. Dreyer, M. Ernstoff, J.C. Marsh, J.R. Bertino, L.R. Whitfield, R. Delap, A. Grillo-Lopez, Cancer Res. 47 (2) (1987) 609.
[119] P.J. Creaven, L. Pendyala, N.J. Meropol, N.J. Clendeninn, E.Y. Wu, G.M. Loewen, A. Proefrock, A. Johnston, M. Dixon, Cancer Chemother. Pharmacol. 41 (1998) 167.
[120] R.G. Foss, C.W. Sigel, J. Pharm. Sci. 71 (1982) 1176.
[121] (a) Anon. USPXXI The United States Pharmacopeia, XXI Revision. Mack Printing Company (1984) pp. 664–665; (b) Anon. USPXXI The United States Pharmacopeia, XXI Revision. Mack Printing Company (1984) pp. 449–450; (c) Anon. USPXXI The United States Pharmacopeia, XXI Revision. Mack Printing Company (1984) pp. 583–584; (d) Anon. USPXXI The United States Pharmacopeia, XXI Revision. Mack Printing Company (1984) pp. 1230–1231.
[122] S.M. Cramer, J.H. Schornagel, K.K. Kalghatgi, J.R. Bertino, C. Horvath, Cancer Res. 44 (5) (1984) 1843.
[123] B. Payet, G. Fabre, N. Tubiana, J.P. Cano, Cancer Chemother. Pharmacol. 19 (4) (1987) 319.
[124] N. Tubiana-Mathieu, S. Monjanel-Mouterde, C. Lejeune, B. Payet, J. Catalin, Y. Carcassonne, J. Cano, Eur. J. Cancer 30A (9) (1994) 1281.
[125] J. Lankelma, E. van der Kleijn, M.J. Jansen, J. Chromatogr. 182 (1) (1980) 35.
[126] K.E. Choi, R.L. Schilsky, Anal. Biochem. 168 (2) (1988) 398.
[127] R.L. Schilsky, K.E. Choi, E.E. Vokes, A. Guaspari, C. Guarnieri, S. Whaling, M.A. Liebner, Cancer 63 (6 Suppl.) (1989) 1018.
[128] C. Vandenbosch, S. van Belle, M. De Smet, G. Taton, V.

Bruynseels, G. Vandenhoven, D.L. Massart, J. Chromatogr. 612 (1993) 77.
[129] E. Brandsteterova, K. Marcincinova, J. Lehotay, A. Zbojova, J. Halko, Neoplasma 40 (4) (1993) 241.
[130] T.B. Hansen, R.A. Wadden, Am. Ind. Hyg. Assoc. J. 49 (2) (1988) 58.
[131] N.A. deWerk, R.A. Wadden, W.L. Chiou, Am. J. Hosp. Pharm. 40 (4) (1983) 597.
[132] P.J.M. Sessink, R.B.M. Anzion, P.H.H. van den Broek, R.P. Bos, Pharm. Weekbl. 14 (1992) 16.
[133] P.J.M. Sessink, N.S.S. Friemel, R.B.M. Anzion, R.P. Bos, Int. Arch. Occup. Environ. Health 65 (1994) 401.
[134] R.M. Mader, B. Rizovski, G.G. Steger, A. Wachter, R. Kotz, H. Rainer, Arch. Environ. Health 51 (4) (1996) 310.
[135] M.J. Tyerman, J.E. Watson, B. Shane, D.E. Schutz, E.L. Stokstad, Biochim. Biophys. Acta 497 (1) (1977) 234.
[136] D.N. Hintze, J.L. Farmer, J. Bacteriol. 124 (3) (1975) 1236.
[137] V.M. Whitehead, Cancer Res. 37 (2) (1977) 408.
[138] V.M. Whitehead, M.M. Perrault, S. Stelcner, Cancer Res. 35 (11 Pt. 1) (1975) 2985.
[139] M.J. Connor, J.A. Blair, Biochem. J. 186 (1) (1980) 235.
[140] R.G. Poser, F.M. Sirotnak, P.L. Chello, Cancer Res. 41 (11 Pt. 1) (1981) 4441.
[141] M.G. Nair, N.T. Nanavati, P. Kumar, Y. Gaumont, R.L. Kisliuk, J. Med. Chem. 31 (1988) 181.
[142] J.J. McGuire, P. Hsieh, J.K. Coward, J.R. Bertino, J. Biol. Chem. 255 (12) (1980) 5776.
[143] R.W. Stout, A.R. Cashmore, J.K. Coward, G.C. Horvath, J.R. Bertino, Anal. Biochem. 71 (1976) 119.
[144] M.J. Barnes, E.J. Estlin, G.A. Taylor, G.W. Aherne, A. Hardcastle, J.J. McGuire, J.A. Calvete, J. Lunec, A.D. Pearson, D.R. Newell, Clin. Cancer Res. 5 (9) (1999) 2548.
[145] C.M. Pfeiffer, J.F. Gregory 3rd, Clin. Chem. 42 (11) (1996) 1847.
[146] G. Fabre, I. Fabre, D.A. Gewirtz, I.D. Goldman, Cancer Res. 45 (3) (1985) 1086.
[147] L.L. Samuels, A. Feinberg, D.M. Moccio, F.M. Sirotnak, G. Rosen, Biochem. Pharmacol. 33 (1984) 2711.
[148] D.W. Fry, J.C. Yalowich, I.D. Goldman, J. Biol. Chem. 257 (4) (1982) 1890.
[149] G. Fabre, I.D. Goldman, Cancer Res. 45 (1) (1985) 80.
[150] P.J. Bagley, J. Selhub, Clin. Chem. 46 (2000) 404.
[151] J. Selhub, Anal. Biochem. 182 (1) (1989) 84.
[152] D.G. Kennedy, H.W. Van den Berg, R. Clarke, R.F. Murphy, Biochem. Pharmacol. 34 (16) (1985) 2897.
[153] G. Fabre, L.H. Matherly, R. Favre, J. Catalin, J.P. Cano, Cancer Res. 43 (10) (1983) 4648.
[154] J. Jolivet, R.L. Schilsky, Biochem. Pharmacol. 30 (11) (1981) 1387.
[155] T. Anzai, N. Jaffe, Y.M. Wang, J. Chromatogr. 415 (2) (1987) 445.
[156] R. Durand, G. Fabre, J.P. Cano, J. Catalin, Q.A. Ahmed, S. Just, J. Appl. Toxicol. 3 (4) (1983) 189.
[157] W. Gibson, G.M. Bisset, P.R. Marsham, L.R. Kelland, I.R. Judson, A.L. Jackman, Biochem. Pharmacol. 45 (4) (1993) 863.
[158] D.G. Kennedy, H.W. Van den Berg, R. Clarke, R.F. Murphy, Biochem. Pharmacol. 34 (16) (1985) 2897.
[159] R.J. Arnold, J.P. Reilly, Anal. Biochem. 281 (1) (2000) 45.