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REVIEW

DRUG LEVEL MONITORING OF ANTIASTHMATIC DRUGS

NORBERT KUCHARCZYK* and FLORENCE H. SEGELMAN

Department of Biochemistry, Wallace Laboratories, Division of Carter-Wallace, Inc., Cranbury, NJ 08512 (U.S.A.)

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1. INTRODUCTION

Bronchial asthma, a major common respiratory disorder which affects 8.5—9 million people in the United States, has a death rate of about 2000 per year [1]. It is, however, more prevalent in children under fifteen years of age than in adults [2]. Antiasthmatic drugs are an important part of the disease management to lower the number of fatalities and reduce suffering during acute attacks, as well as to help prevent or to lower the number and frequency of future asthmatic attacks.

According to their known or assumed mode of action, antiasthmatic drugs are classified as bronchodilators, anti-inflammatory steroids, and asthma prophylactics [3, 4]. Bronchodilators are by far the most numerous and widely used class and are further subdivided into β-adrenergic stimulants, phosphodiesterase inhibitors, and anticholinergics. Significant advances in asthma therapy have recently been achieved by the introduction of new drugs, thus leading to a wider choice of therapeutic agents [5]. Not all of the new introductions are included in this review because, for some substances, chromatographic assays suitable for use in drug level monitoring (DLM) have not been published during the review period of 1978—1983.

The abbreviations used in this review are given in Table 1. The structures and names of antiasthmatic drugs reviewed in this paper are shown in Table 2.

TABLE 1

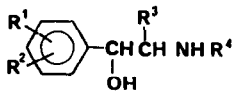
LIST OF ABBREVIATIONS WITH DEFINITIONS

BSTFA	Bis(silyl)trifluoroacetic acid
cAMP	Adenosine monophosphate
CI	Chemical-ionization mode
C.V.	Coefficient of variation, intra-assay precision, within-run, within-day
C.V.(R)	Coefficient of variation, inter-assay precision, reproducibility, day-to-day or between-run variability
DEHP	Di-(2-ethylhexyl) phosphate
DLM	Drug level monitoring
DSCG	Disodium cromoglycate
EI	Electron-impact mode
GC	Gas chromatography
HFBA	Heptafluorobutyric anhydride
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
IA	Immunoassay
ID	Isotope dilution
MS	Mass spectrometry
RIA	Radioimmunoassay
RP	Reversed phase
TLC	Thin-layer chromatography
UV	Ultraviolet

TABLE 2

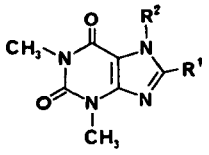
STRUCTURE AND NAMES OF ANTIASTHMATIC DRUGS

A. β-Adrenergic stimulants



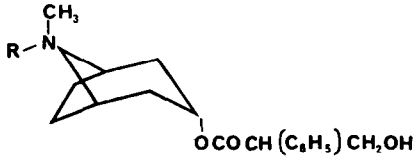
Name	R^1	R^2	R^3	R^4
Albuterol (salbutamol)	3-CH_2OH	4-OH	-H	*tert.*-C_4H_9
Terbutaline	3-OH	5-OH	-H	*tert.*-C_4H_9
Fenoterol	3-OH	5-OH	-H	1-(4-hydroxyphenyl)-2-propyl
Isoproterenol (aludrine)	3-OH	4-OH	-H	iso-C_3H_7
Metaproterenol (orciprenaline)	3-OH	5-OH	-H	iso-C_3H_7
Isoetharine (etyprenaline)	3-OH	4-OH	-C_2H_5	iso-C_3H_7
Epinephrine (adrenaline)	3-OH	4-OH	-H	-CH_3

B. Phosphodiesterase inhibitors



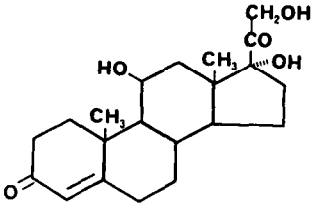
Name	R^1	R^2
Theophylline	-H	-H
Propoxyphylline	-H	2-hydroxypropyl
Dyphylline (dyprophylline, glyphylline, lufyllin)	-H	2,3-dihydroxypropyl
Bamifylline	-benzyl	2-(N-ethyl-N-2-hydroxy-ethylamino)ethyl

C. Anticholinergics

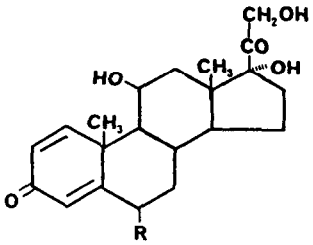


Name	R
Atropine (*d,l*-hyoscyamine)	-H
Ipratropium	-iso-C_3H_7

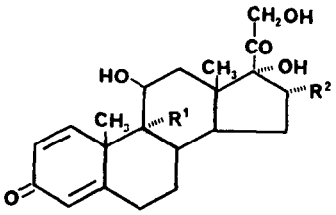
D. Steroids



Cortisol (hydrocortisone)

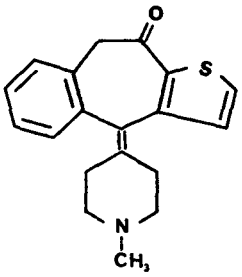


Name	R
Prednisolone (Δ^1-dehydrocortisol)	-H
Methylprednisolone	$-CH_3$

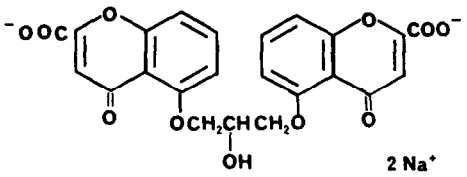


Name	R^1	R^2
Triamcinolone	-F	-OH
Dexamethasone (hexadecadrol)	-F	CH_3

E. Prophylactic agents



Ketotifen



Disodium cromoglycate (FPL 670, cromolyn sodium)

2. CLINICAL IMPORTANCE OF DRUG LEVEL MONITORING IN THE TREATMENT OF ASTHMA

In the last few years the importance of DLM for the clinician has increased dramatically in many fields of therapy. This is evident from comprehensive reviews on this subject recently published by Pippenger and Massoud [6], by Evans et al. [7], and by Rylance and Moreland [8]. One of the most illustrative cases is that of the antiasthmatic drug theophylline where progress in therapeutic drug monitoring has significantly increased efficacy and contributed to its safe use. The clinician's need for a reliable assay to monitor drug levels is almost mandatory for drugs with a narrow therapeutic index such as theophylline or isoproterenol. It is less important for drugs that have few toxic side-effects or have a short half-life such as epinephrine. Whenever efficacy of treatment is of concern, the clinician has to have proof that the patient is complying with the prescribed dosage regimen because the compliance rate in taking antiasthmatic medication is reported to be only 46% [9]. Consistent drug levels in the circulation are proof of compliance.

A useful and reliable assay produces results which the clinician or pharmacokineticist can fully understand and trust. Such an assay can only be developed if sufficient information on the biotransformation, pharmacokinetics, pharmacodynamics, and toxicity of the drug is available in all patient popula-

tions. This is usually not the case in the early phases of drug development or clinical use. Nevertheless, assays have to be developed and are published even without the availability of the above mentioned essential information [10]. Therefore, the criteria for evaluation of the first assays during drug development are, of necessity, less strict.

In our review the criteria for evaluation of methods used in DLM are precision and accuracy as defined elsewhere [11]. Sensitivity is expressed in quantitative terms as limit of detection, and selectivity means the absence of other interfering endogenous or exogenous analytes. Consideration is also given to the amount of sample needed, the analysis time, and the complexity of the procedure. A useful method has to be transferable to different environments and should be reproducible whenever time, equipment, and operators change (day-to-day variability). Data on storage stability of the drug in the biological matrix, reagent and standard solutions stability, column life, solvent quality, etc., increase the value of any DLM-related publication.

Guidelines for methods used in therapeutic drug monitoring have been proposed very recently in the United States by the National Committee for Clinical Laboratory Standards [12] in order to "ensure that clinically valid results are consistently obtained from immunochemical or chromatographic methods" and to establish "common performance criteria for precision, specificity, sensitivity, and accuracy". We have found the suggested protocol to be quite useful in method evaluation and validation. It emphasized the need to cover the full therapeutic range of concentrations and the use of standard reference samples using the biologic matrix of concern. Further, it suggested the determination of within-run and total precision for twenty days, each day in duplicate, as well as the accuracy at five different concentrations throughout the range. In 1978 Chamberlain [13] complained that "few authors reported precision, accuracy, sensitivity, etc., in a quantitative form or gave much consideration to such aspects in the evaluation of the method". According to our review this is still true for many of the assays for antiasthmatic drugs published in the past five years.

3. CHROMATOGRAPHIC ASSAY PROCEDURES FOR ANTIASTHMATIC DRUGS

3.1. β-Adrenergic stimulants

3.1.1. Albuterol

The physical properties, metabolism, absorption, and excretion as well as methods of analysis for this drug have been reviewed in 1981 by Aboul-Ehein et al. [14]. It is given orally or in aerosol form. Its metabolites include the 4-O-sulfate ester, which is a major urinary metabolite in man [15], and an unkown metabolite in plasma [16]. The half-life of this drug was reported to be 6 h, and the plasma levels after 16 mg varied from a low of less than 1 ng/ml at 24 h to a high of 26 ng/ml at 6 h [17]. Walters et al. [18] reported plasma levels in asthmatic patients ranging from 0 to 0.6, 0 to 2.3, and 1.4 to 3.6 ng/ml at 30 min after an oral dose of 1.5, 3, and 7.5 mg. For asthmatic children 2 mg given rectally or 0.2 mg inhaled led to 1-h plasma levels of 1.2 to 12.3 ng/ml or 0.5 to 1.6 ng/ml, respectively [19]. The range of drug concentration in plasma in man based on the literature is 0.5—26 ng/ml.

The classical method for albuterol is the gas chromatographic—mass spectrometric (GC—MS) procedure of Martin et al. [20], already reviewed elsewhere [14]. Since then Plavsic [21] suggested a radial high-performance thin-layer chromatographic (HPTLC) procedure on silica gel with ethanolic ammonia as the solvent and detection using 2,6-dichloroquinone chlorimide. This semiquantitative procedure for the drug in urine gave a limit of detection 0.5 mg/l. This method might be useful in monitoring patient's compliance. The same author compared different absorption techniques for albuterol on XAD-2, XAD-4, charcoal, Norit A, Amberlite CG50, and medical gauze [22]. He found the ion-exchange resin the most suitable at pH 6 using elution with methanolic ammonia. However, no quantitative values were reported. The problem of extraction of phenolic ethanolamines from aqueous media has been studied by Brandts et al. [23] who recommended di-(2-ethylhexyl) phosphate (DEHP) as the ion-pairing reagent in combination with various solvents to be optimal for good drug recovery; e.g. 3-methylbutanol gave 70% drug recovery. The same group published preliminary results [24] on an improved method that extracted samples on C_{18} Bondelut® or Sep-Pak® cartridges followed by derivatization with bis(silyl)trifluoroacetic acid (BSTFA) prior to GC—MS chemical ionization (CI) quantitation by a method published earlier for terbutaline [25]. The drug recovery from serum was 80% over the range 2—20 ng/ml with a limit of detection of 1 ng/ml. This was a significant improvement. In another report an electrochemical detector with a rotating disc electrode and a bimodal column high-performance liquid chromatographic (HPLC) system was used for human plasma albuterol determination by Oosterhuis and Van Boxtel [26]. They used Sep-Pak for extraction and relatively complex home-made equipment; 95—101% recovery was reported for the range 2.5—20 ng/ml with a coefficient of variation (C.V.) of 9.8% at 4.7 ng/ml. Based on the signal-to-noise ratio a limit of detection of 0.5 ng/ml was claimed. No interference from theophylline, prednisone, and beclomethasone as well as some other drugs was seen. Tanner et al. [27] described the computer automation of their original GC—MS procedure [20] by inclusion of the DuPont (U.K.) Prep I automatic sample processor for XAD-2 column elution and a Pye Unicam Type S8 autoinjector coupled to the gas chromatograph. The recovery for the range 0—20 ng/ml was 93 ± 2% (with radiolabeled drug), whereas spiked plasma samples containing 1.07 ng/ml gave an accuracy of 8%; at this concentration the C.V. was 9% and the limit of detection was claimed to be 0.25 ng/ml. A drug-derived peak, not interfering with albuterol, was observed. This method, when compared with the above HPLC—electrochemical detection assay [26], gave consistently higher results. The effect of amine modifiers (tertiary and quaternary ammonium compounds) on peak shape of the drug on reversed-phase (RP) columns (Zorbax® CN, Zorbax® ODS) was studied by Eggers and Saint-Joly [28]. They claimed that tetrapropylammonium hydroxide was effective in eliminating tailing of the drug, although from the literature it was not clear that tailing of albuterol was a serious problem. Hutchings et al. [29] used the Perkin-Elmer 3000 fluorescence spectrometer (excitation at 230 nm, emission at 309 nm) to quantitate the drug by HPLC in the range 0—100 ng/ml. A relatively simple clean-up procedure used ion-pair extraction with DEHP in chloroform and chromato-

graphy on a 5-μm Zorbax RP column with 8% acetonitrile in 0.15% phosphoric acid to get 84 ± 3% recovery; this was contrary to ion-pair extraction results of the Utrecht group [23]. Very clean chromatograms resulted using 1 ml of plasma with a 15-min analysis time. From the signal-to-noise ratio of 2:1 the limit of detection was estimated at 1 ng/ml, the C.V. at 100 ng/ml was 2.5% and at 5 ng/ml 3.2%, and the C.V.(R) at 40 ng/ml was 11%. No interference was encountered from theophylline, betamethasone, dexamethasone, and other commonly used drugs. This method appears to be the most simple and useful in DLM as it has the capacity to lower the limit of detection further by increased sample size and by choice of a lower excitation wavelength. For simultaneous determination of albuterol with terbutaline see *3.1.2. Terbutaline.* No other non-chromatographic methods applicable to DLM have been published for albuterol.

3.1.2. Terbutaline

Terbutaline has been administered by all possible routes. In man it is not metabolized, except for the formation of the sulfate conjugate which, besides the unchanged drug, is the only excreted major metabolite [16]. Therefore, no interfering metabolite would be expected in the circulation of man. According to the literature [25, 30—36] terbutaline levels ranged from 0.6 to 33.9 ng/ml (see Table 3), and the therapeutic range appeared to be 2—10 ng/ml. Above 10 ng/ml some toxic side effects were observed.

One of the first methods for quantitation of drug in serum and urine at therapeutic levels of 1—20 ng/ml was published by Leferink et al. [25]. In this paper ion-pair extraction with DEHP in ethyl acetate was used and, as previously shown by the author [23], yielded 87% recovery. After extraction the residue was silylated with BSTFA and analyzed by GC—MS in the multiple-ion detection mode. d_6-Terbutaline was used as internal standard and the ions *m/e* 426, 442, 432, and 448 in the CI mode were measured. GC separation was carried out on a 3% OV-1 (Gas Chrom Q) column at 165°C with helium or methane as carrier gas. Serum (1 ml) or 0.1 ml of urine was analyzed. For samples with more than 100 ng of drug the electron-impact (EI) mode was also

TABLE 3

TERBUTALINE PLASMA/SERUM LEVELS IN HEALTHY AND ASTHMATIC SUBJECTS

Subjects	Dose (mg)	Route	Drug levels* (ng/ml)	Reference
Normals	0.5, 5	s.c., p.o.	7.5 at 0.5 h, 1 at 6 h in serum**	30,33
Atopic asthmatics	0.5, 5	s.c., p.o.	6.9 at 0.5 h, 1 at 6 h in serum**	30,33
Normals	0.25	i.v. infusion	max. = 10 in plasma**	31
	5 t.i.d.	p.o.	max. - 5.5 at 2 h in plasma**	31
Child	45 (overdose)	p.o.	33.9 at 3 h in plasma	32
Normals and asthmatics	5.26—5.9 μg/kg	s.c	7.2 at 0.45 h in serum	34
	55.3 μg/kg	p.o.	max. = 2.3—5.03 at 3.3 h in serum	34
Asthmatic children	0.075 mg/kg	p.o.	max. = 1.3, min. = 0.6 at 5 h in plasma	35
	0.25 mg/kg		max. = 2.7, min. = 1.7 at 5 h in plasma	
Severe-to-moderate asthmatics	5	p.o.	max. = 3.7—6.5 at 2 h in plasma min. = 2.1 at 7 h in plasma	36
Asthmatics	5	p.o.	max. = 9.4 at 2 h, min. = 1.0 in serum	25
	0.25	s.c.	max. = 5.6 at 0.5 h, min. = 1.2 at 3 h in serum	

*Max. = maximum; min. = minimum.
**Estimated value from graph.

suitable. No limit of detection was estimated, and the C.V. was calculated from nine replicates at 2.5 ng/ml of serum to be 8%. Recovery was 80 ± 6%. Albuterol, isoetharine, fenoterol, and metaproterenol did not interfere. This was also the first paper [25] to report therapeutic drug serum levels in man (see Table 3). The authors adapted this method for DLM in post-mortem lung, liver, heart, muscle, kidney, and serum samples [37]. Recoveries were 60 ± 5%, the limit of detection was 0.75 ng/ml with 0.5-g samples, and the range was 2—50 ng per 0.5 g tissue. The selectivity of this assay appears to be very high.

Clare et al. [38] also developed a GC—MS assay for the drug in plasma and urine in the range 0—15 ng/ml. GC was carried out on 3% OV-17 on Gas Chrom Q. Single-ion monitoring at *m/e* 355 was used for both the drug and the internal standard, a terbutaline analogue. A 4-ml volume of plasma was needed for the clean-up procedure consisting of ethyl acetate extraction at pH 9.8 and re-extraction into 0.1 *M* hydrochloric acid followed by a complicated derivatization step. The drug recovery was 71% and the limit of detection was 0.3 ng/ml. The C.V. determined at 0.3, 5, and 14 ng/ml was 14.3%, 9.5%, and 8.3%, respectively, and the accuracy ranged from 6.7% to 7.9%. This method allowed measurement of unconjugated and total drug (after enzymatic hydrolysis). However, no data were included for its use in urine. For this method the lengthy sample preparation would be a disadvantage.

Martin et al. [39] described briefly a GC—MS assay for human plasma in the range 0—10 ng/ml using 2 ml of sample with an estimated limit of detection of 0.25 ng/ml. At the low level of 1 ng/ml the precision was 4.1%. This paper [39] referred to the method for albuterol [20] and claimed 70 ± 5% recovery of drug. Also, from the tracing the limit of detection for the drug appears unrealistically low and the use of the antiasthmatic drug, albuterol, as the internal standard might limit the use of this method in DLM.

An assay suitable for the low range 0.1—5 ng/ml terbutaline in plasma was developed by Jacobsson et al. [40]. It used ion-exchange resin AG50W for a simple clean-up (as recommended by Plavsic [22]) contrary to the ion-pair extractions of other procedures. The solution was then extracted with *tert.*-butanol and derivatized with BSTFA in pyridine. The GC—MS quantitation was carried out on a 3% OV-1 on Varaport 30 column with helium as carrier gas. The mass spectrometer was used in the CI mode with ammonia as reagent gas. The *m/e* 442 and 448 peaks were monitored (d_6-terbutaline as internal standard). The C.V. was 8.6% at 0.1 ng/ml and 3.4% at 5 ng/ml, C.V.(R) at 3.26 ng/ml was 6%, recovery of drug 80—85%. The only disadvantage of this otherwise excellent method [40] was the 4 ml of plasma needed, which could be a problem for pediatric use. Its advantages, however, were a simple clean-up, fast derivatization, and excellent precision for low-level determinations. A minor modification consisting of a change in the ion-pair clean-up allowed simultaneous determination of albuterol to 0.3 ng/ml [41]. This further increased the usefulness of the method. A semi-quantitative HPTLC method for the drug in urine in the range 0—1.6 μg/ml was developed by Plavsic [21] which was analogous to that for albuterol. A GC—MS method already reviewed for albuterol [24] also allowed determination of terbutaline in serum in the range 2—20 ng/ml with a limit of detection of 1 ng/ml.

The first HPLC procedure with electrochemical detection was published by

Bergquist and Edholm [42]. It covered the range 5—50 pmol/ml (1.13—11.3 ng of base per ml) using 2 ml of plasma. The procedure consisted of an ion-exchange resin clean-up and on-line enrichment on Sep-Pak C_{18} packing with back-flushing onto the analytical Nucleosil C_{18} column. A commercial electrochemical detector with a glassy carbon electrode led to sufficient sensitivity. The C.V. was 8% at the lowest and 0.3% at the highest concentration, and drug recovery was 91 ± 2% at 50 pmol/ml. The results of assays of clinical plasma samples obtained with this method [42] were consistently higher (2—33%) than those of the GC—MS assay developed by Jacobsson et al. [40]. Because GC—MS assays are still more expensive, this relatively simple HPLC procedure appears to be more feasible for routine DLM in the therapeutic range.

3.1.3. Fenoterol

Very little information on the disposition of this drug in man as well as on the extent of its use in asthma treatment was found in the literature. According to the study of Rominger and Pollman [43], using ^{3}H-labeled drug, plasma levels after an i.v. dose of 0.9 μg/kg varied from a maximum of 1.3 ng/ml to a minimum of 0.5 ng/ml; the maximum after an oral dose of 90 μg/kg was 41 ng/ml and the minimum was 7 ng/ml.

The method of Leferink et al. [24] for determination of terbutaline and albuterol was also used for the determination of fenoterol (for details, see ref. 24). According to the authors, the limit of detection of 1 ng/ml was not sufficient for measuring the much lower therapeutic levels.

3.1.4. Isoproterenol

The serious side-effects of this drug have limited its use as an antiasthmatic drug since the recent introduction of more specific β-adrenergic stimulators. Similarly, as for the previous drug fenoterol, the information on the disposition of isoproterenol in man was limited. The drug was partly metabolized to its O-methyl derivative after i.v. administration; when given orally, almost all of it was excreted as a conjugate of the drug [44]. At a dose of 0.44 μg/kg infused over 30 min, levels of 0.66 ng/ml were detected by radioactivity. Following an oral dose of 0.2 mg/kg a maximum of approximately 450 ng/ml was detected in plasma.

Only two methods suitable for the use in DLM have, to our knowledge, been published for this drug. Kishimoto et al. [45] developed a relatively simple HPLC method for direct determination of the drug in deproteinized human plasma (1—240 pmol/ml) or urine (20 pmol/ml to 4.8 nmol/ml). They used a Zipax-SCX ion-exchange column and a monobasic sodium phosphate buffer, pH 4.3, as mobile phase. Post-column derivatization to a fluorescent trihydroxyindole by oxidation with $K_3Fe(CN)_6$ was used for very specific detection in a spectrofluorometer (excitation at 400 nm, emission at 510 nm). The major metabolite O-methylisoproterenol did not interfere and no other interfering substances were found in plasma or urine. The limit of detection in plasma was 0.2 pmol (1 pmol = 0.211 ng). The C.V. at 1.9, 4.8, and 9.4 pmol in plasma was 2.3, 2.1, and 2.1%, respectively. The C.V.(R) at 1.8 and 9.4 pmol was 5.6% and 4.3%. The recovery from plasma ranged from 87% to 91% and from urine from 100% to 103%. Excellent precision and accuracy were achieved even though no internal standard was used.

Causon et al. [46] reported an assay for the inactive *d*-enantiomer of isoproterenol in human plasma and urine by HPLC with amperometric detection. Urine or plasma samples containing the drug sulfate conjugate were first hydrolyzed with hydrochloric acid and then batch-adsorbed onto alumina similarly as in the procedure of Deyl et al. [47] published earlier. After elution with 0.1 *M* orthophosphoric acid the eluate was analyzed on an Ultrasphere C_{18} column with citrate—phosphate buffer, pH 6, containing 3% methanol. N-Methyldopamine was the internal standard. The C.V. at a high concentration of 632.6 ng/ml in plasma was 2.6% and in urine 3.1% with a C.V.(R) of 4.6% and 7.1%, respectively. The recovery was 71% for isoproterenol and 68% for the internal standard. At a detector sensitivity setting of 10 nA the limit of detection was 0.5 ng/ml and this could be enhanced by higher amplification. In our opinion this assay could well be used for the drug, *l*-isoproterenol, with its simple clean-up procedure adaptable for routine use or automation.

3.1.5. Metaproterenol

This drug has replaced isoproterenol during the past few years. It is used by inhalation or by the oral route (20 mg three times a day) for chronic disease management. The drug is absorbed and metabolized by conjugation to only one metabolite, the 3-O-sulfate ester [48]. No data on plasma/serum levels of the drug have been published to our knowledge.

The only chromatographic assay for this drug in biological fluids was that of Macgregor et al. [48]. The briefly described HPLC procedure was developed for determination of drug in human urine in the range 0.5—50 μg/ml with good precision and reproducibility (C.V. 10%). This assay would not be suitable for use in routine DLM.

3.1.6. Isoetharine

The information available on the disposition and blood levels of the inhalation bronchodilator isoetharine was scant [49].

An HPLC assay developed for the drug in rat plasma was published by Park et al. [50]. Plasma samples (25—150 μl) were extracted with the ion-pairing reagent DEHP in benzene and re-extracted into the aqueous phase after acidification. The aqueous phase was injected onto a 5-μm RP Ultrasphere-ODS column with buffered 0.1 *M* sodium sulfate (pH 3.0)—methanol (87.5:12.5) as mobile phase.

The drug and its analogue colterol as the internal standard were quantitated with an electrochemical detector. The limit of detection was 0.92 ng/ml. The recovery of drug and internal standard was 76—83%, and the C.V. varied from 2.1% to 5.1%. The accuracy was better than 5%. Although the assay was intended for rat plasma we considered this paper to be a good example of a presentation of a DLM assay. It was the only chromatographic procedure for the drug and should be adaptable for DLM in man.

3.1.7. Epinephrine

This endogenous catecholamine has an important function in the regulation of the bronchial muscle tone by increasing the level of intracellular adenosine monophosphate (cAMP). However, plasma/serum levels of epinephrine seem to

vary widely depending on emotional or physical stress, environmental considerations, disease state, general nutrition and food intake, circadian rhythm, metabolism, and many other factors. Levels of 10—1000 pg/ml have been reported [51—55]. No examples of DLM in asthmatics dosed with epinephrine have been found in the literature. As observed recently by Brown [56], the overwhelming majority of catecholamine assays in hospitals were performed for research purposes.

Administered epinephrine exhibits a very short half-life of 1—2 min and then the blood levels return to normal steady state within 5—10 min [52]. The very slight increases of blood levels coupled with overall analytical problems preclude routine DLM of this rapidly acting drug.

We also felt that discussion of recent advances in catecholamine assays was, by the great number of publications in this field, outside the scope of this review. However, for interested readers an excellent review of the physiological regulation and methods of determination of catecholamines (including epinephrine) has been published in 1983 by Barrand and Callingham [57]. Similarly, Kringe et al. [58] discussed the challenges in development of a routine catecholamine assay.

3.2. Phosphodiesterase inhibitors

3.2.1. Theophylline and salts

Recent publications discuss in detail the new knowledge pertinent to theophylline pharmacokinetics [59—62] and pharmacodynamics [63—66] which have advanced the treatment of both acute and chronic asthma. In very general terms theophylline is poorly soluble in water (pH = 7) and the more-water-soluble salts with ethylenediamine, choline, calcium salicylate, and sodium glycenate were formulated. However, increased solubilities did not improve theophylline absorption. Theophylline may be administered orally, rectally, and intravenously in a variety of dosage forms. In the normal adult orally administered theophylline has a half-life of about 4.5 h. This parameter is highly variable depending upon underlying disease states, concomitantly administered medications, oral dosage form used, and the age of the patient (children, half-life about 3.6 h, neonates about 15 h) as well as many other factors. Peak blood concentrations are achieved at about 2 h post-dosing. Related to the above is metabolism of theophylline in the liver where it is transformed mainly to 1,3-dimethyluric acid as well as to 1- and 3-methylxanthine and 1-methyluric acid.

Paramount to the pharmacodynamics of theophylline is its narrow therapeutic range, 10—20 μg/ml, which is the optimal blood level range to prevent the symptoms of chronic asthma. Above this range toxicity occurs which can be minor, such as the caffeine-like side-effects, or in extreme cases cardiac arrest or seizures. Monitoring of theophylline levels by rapid, sensitive and accurate assay methods becomes critical in the management of the asthmatic.

More recent reviews [67—70] on the assay of theophylline in biological fluids describe the vast improvements in methodology over the earlier classical spectrophotometric procedures. The new literature (1978—1983) continues

to provide improved methodologies particularly in HPLC and immunoassays containing a variety of labels both with and without radioactivity. In addition an article by Yosselson-Superstine [71] points to a more recent emphasis in DLM, i.e., common drug—drug assay interferences including those associated with the assay of theophylline.

As shown in Table 4 (subsection *a*), and as reviewed below, the new HPLC assays often report a comparison methodology including, in many cases, one of a non-chromatographic type.

TABLE 4

SUMMARY OF COMPARISON PROCEDURES FOR THEOPHYLLINE ASSAYS

Primary type of method	Instrumentation	Reagent kit	Comparison methods	Reference
a. HPLC versus primarily non-chromatographic procedures				
HPLC	—	—	EMIT	72
HPLC		—	EMIT, HPLC	73
HPLC	—	—	RIA	76
HPLC	—	—	EMIT, HPLC	77
b. Non-chromatographic versus chromatographic procedures				
SLFIA	Optimate	Optimate	EMIT, HPLC*, GC*	78
SLFIA	Fluorostat	TDA	HPLC**	79
SLFIA	Aminco fluorocolorimeter		RIA, EMIT, HPLC	80
SLFIA	Fluorostat	TDA	HPLC***, TDA, EMIT	83
FPIA	Abbott TDx	TDx	HPLC	84

*In-house developed, no description in detail in this paper.
**Instrumentation and brief description only in this paper.
***No description given.

The method of Ou and Frawley [72] intended for neonate specimens involved a 5-μm C_{18} RP column. Using 50 μl serum they achieved a limit of detection of 0.5 μg/ml with an overall assay time of 6 min (ten samples per h). No interference was observed with fourteen other drugs including xanthines (8-chlorotheophylline, dyphylline, 3-methylxanthine and theobromine), various cephalosporins and different sulfa antibiotics as well as other drugs. The recoveries for theophylline, caffeine, and the internal standard, 3-hydroxytheophylline, were 98—101% over the range 10—20 mg/l. The C.V. values for all drugs were about 3%. Comparison of this procedure with the EMIT assay showed excellent correlation. The only drawback to the HPLC method was the interference due to paraxanthine (1,7-dimethylxanthine), a metabolite of caffeine. For the adult patients ingesting large amounts of caffeinated beverages this method would be unsuitable.

However, Kabra and Marton [73] published a procedure which did resolve paraxanthine from theophylline using modifications in the injector (6-μl loop), detector (2.4-μl microcell), and narrow-bore tubing (0.25 mm). This system used RP C_{18} column packing, acetonitrile—PO_4 buffer (pH 3.6) as mobile phase (9.5:90.5), oven temperature of 50°C with UV monitoring at 273 nm. A large number of drugs did not interfere; however, dyphylline would probably

interfere with the theophylline assay since their retention times were 0.88 and 0.91 min, respectively. Other xanthines, such as caffeine, theobromine, 8-chlorotheophylline, 2-methylxanthine, and 3-hydroxyethyltheophylline (the internal standard) were all well resolved. The C.V.(R) and C.V. were excellent (2—3.5% and 2—2.5%, respectively) with drug recovery ranging from 97% to 102% over the range 4—50 μg/ml giving a limit of detection of 0.5 μg/ml. Accuracy was assessed by comparing the linear regression parameters of this HPLC method [73] to those of the EMIT and RIA assays [74] and another published HPLC procedure [75] and the comparisons were favorable. This paper claimed that twenty samples per h could be completed using as little as 25—100 μl of serum. However, modifications of an existing HPLC unit would be required which might limit the utility of this assay.

Van Aerde et al. [76] also resolved paraxanthine from theophylline on 5 μm particle size silica using a mobile phase consisting of chloroform—dioxane—formic acid (95.5:4.5:0.01) with UV monitoring at 273 nm. A 100-μl volume of plasma containing the internal standard, 3-isobutyl-1-methylxanthine, and saturated ammonium sulfate solution was extracted using a mixture chloroform—2-propanol (95:5). This gave 91% recovery of theophylline and 99.8% for the internal standard with 10 ng/ml as the limit of detection. Spiked plasmas at 1, 8, and 15 mg/l gave a C.V. of 2.5, 2.2, and 2.7%, respectively. Calibration curve parameters remained virtually unchanged during a one-month interval of repeated comparisons. Accuracy was further assessed by direct comparison of theophylline levels determined in 100 samples by radioimmunoassay (RIA) (Gammadab®) measurements and gave excellent correlation as tabulated in the publication [76]. Caffeine, theobromine, and 1- and 2-methylxanthines or methyluric acids were presumably not extracted or were retained on the column so that they did not interfere with theophylline or the internal standard. This method [76] would be more suitable for pharmacokinetic analyses or for pediatric samples. Other therapeutic xanthine drugs were not considered in this assay, which is a drawback for this procedure. Bock et al. [77] also reported on the RP-HPLC separation of theophylline and paraxanthine on 3-μm silica solid support. This was a very rapid automated serum theophylline determination (10 min total assay time). Samples containing the internal standard, 8-chlorotheophylline, were treated with zinc sulfate and methanol and 10 μl of each supernatant were analyzed. HPLC assay [77] results were compared to the EMIT assay results and found to correlate well with $r = 0.96$. Repeat assays at the 15 μg/ml level gave a C.V. of 2.7% and C.V.(R) of 4.6%. Other drug substances tested for interference included theobromine and eleven other non-xanthines. This was rated as the overall best HPLC procedure for routine DLM of theophylline.

The majority of the current literature on theophylline immunoassays were comparative in nature, i.e., how did they perform one against another or to chromatographic (gas or liquid) methodologies. A variety of non-radioisotopic labels and procedures are available, including the use of enzymes, fluorescent, or other immunolabels: enzyme-multiplied immunoassay (EMIT); ligand displacement immunoassay (LIDIA®); substrate-labeled fluorescent immunoassay (SLFIA) such as the Ames TDA®; fluorescence polarized immunoassay

(FPIA) such as the Abbott TDx™; apoenzyme reactivation immunoassay system (ARIS) as well as the newer competitive immunoinhibition assay as measured in the Beckman ICS rate nephelometer.

In Table 4 (part *b*), a summary of the major differences among the immunochemical procedures reviewed are given which includes the instrumentation and the comparison methods used for correlation of these procedures. Li et al. [78] used the Optimate™ fully automated clinical analyzer system and reagents for theophylline and eight other drugs. Calibration curve data obtained initially and after two-week storage showed a slightly higher C.V. of 7.5% versus 4.1%. The limit of detection of this assay method was 0.8 μg/ml for theophylline. Correlation of this automated method and the manual equivalent was excellent as was the correlation ($r > 0.97$) with the HPLC, GC, and EMIT reference procedures. Samples (spiked) were run which used the "over-range" mode and when compared to the stated weighed concentrations came within 3—7% (precision). Sample throughput of 92 per h was achieved and abnormal sera (hemolyzed, lipemic, or ecteric) did not alter the theophylline assay. Computer-controlled operations and small sample size requirements (50 μl) were other advantages. No cost per sample or reagent problems were discussed, but this procedure would be extremely useful in the large clinical laboratory setting.

Similarly, James et al. [79] found comparable results for the TDA Fluorostat technique to a HPLC method. In his opinion the TDA method would be improved with the addition of a 5 μg/ml calibrant. Overall, the cost for the TDA or HPLC systems were about the same, but the TDA system offered flexibility and assay-on-demand capability over HPLC. Actual clinical specimens analyzed by both methods gave similar results with the TDA calibrant showing a slight positive bias in the range 10—20 μg/ml. Castro and Steele [80] further evaluated the fluorescence immunoassay method (assumed TDA) and compared it to RIA [81], EMIT, and HPLC [82] procedures for theophylline on specimens obtained from acute-care patients which would have potential interferences in their plasma owing to concomitant medication and disease states. All methods compared favorably except for the HPLC positive bias of 1.5 μg/ml, which was attributed to other xanthines, perhaps paraxanthine. However, the authors commented that each method had at least one outlier (> 30% difference from other methods) and all methods were deemed suitable for routine clinical use.

A modification of the TDA procedure is given by Davis and Marks [83] in which the incubation time for the reaction was shortened to 5 min and calibration curve storage was extended to three weeks without compromising assay accuracy as compared to the non-modified TDA procedure and two other methods (Table 4). Abnormal plasma constituents did not interfere with the theophylline assay but the stored calibration curve must be "corrected" or normalized and the same incubation temperature must be used throughout the curve storage interval. The cost- and time-saving advantages were apparent but are not discussed in this paper.

A theophylline level assay using FPIA TDx procedure [84] was found to compare favorably with an HPLC method [82]. This assay was fully automated and clinical serum specimens were used in the correlation study. Recovery of drug from a variety of normal and abnormal sera was about 97% with a

limit of detection of 0.4 μg/ml using a typical 1.8-μl sample size. A slight positive bias was observed for theophylline assays in the presence of increased serum bilirubin concentrations. Calibration curves for theophylline were repeated every 1.5 weeks. Overall, the procedure appeared rapid and reliable enough for routine theophylline level determinations. As pointed out, however, in another reference [85] theophylline levels may be falsely high in the serum of uremic patients whenever analyzed by the TDx system.

3.2.2. Dyphylline

Dyphylline is rapidly absorbed orally and gives mean peak serum levels (7.4 μg/ml) in about 30 min for a single 400-mg dose [86]. Even at higher single oral doses of about 1500 mg [87] it has a short half-life of about 2 h with 84% urinary excretion of intact drug in 24 h. Intravenous administration showed two-compartment pharmacokinetics. Jarboe et al. [88] found that the dyphylline distribution ratio between serum and milk in lactating women was 2.08.

Wenk et al. [89] described a procedure for the simultaneous determination of theophylline as well as two derivatives, dyphylline and propoxyphylline in 0.5 ml serum or plasma over the concentration range 0.25—12 μg/ml with an estimated recovery (10 μg/ml level) for all analytes of 95—96% and 90% for the internal standard, 8-chlorotheophylline. Reported limits of detection were 0.2, 0.25, and 0.1 μg/ml for dyphylline, propoxyphylline and theophylline, respectively. Serum specimens stored at 40°C or room temperature for up to seven days were stable. Excellent precision and accuracy values were given for all compounds within-day and day-to-day and concomitant medication such as penicillin G, phenobarbital, and six other common drugs did not interfere. The chromatography was carried out on 5-μm ODS RP packing with a mobile phase of acetate buffer, pH 5.2—acetonitrile—methanol and UV monitoring at 274 nm. This assay [89] was recommended for routine therapeutic monitoring of the above xanthines. The pH of the mobile phase had to be monitored since the retention time of dyphylline was sensitive to this factor. The advantage to this procedure over a previous one [72] was that no modifications of an existing HPLC unit would be required.

Valia et al. [90] reported an HPLC method to simultaneously quantitate dyphylline and theophylline in the range 2.5—50 μg/ml using a 10-μm RP C_{18} column with acetate buffer—acetonitrile (94:6) as the mobile phase and UV monitoring at 274 nm. Following a simple trichloroacetic acid treatment of 1.0 ml plasma, injections of 25 μl of supernatant gave 95—99% recovery for dyphylline and 95—116% for theophylline. The limit of detection was 0.4 and 1.0 μg/ml, respectively. The C.V.(R) ranged from 1.8% to 4.8% for dyphylline and from 5.3% to 6.2% for theophylline. A run time of about 10 min would be required per sample (60 samples per 12-h overnight run).

Paterson [91] used an RP 10-μm C_{18} column and phosphate buffer, pH 3.0—methanol (3:1) for determining dyphylline. Linearity was achieved only to 20 μg/ml in serum ($r > 0.99$) with precision assessed over the 2.5—10 μg/ml range [C.V. 4.7—5.3%; C.V.(R) 5.3—7.2%]. Drug recovery was 92—99%, and the limit of detection was 1 μg/ml for dyphylline. The sample preparation steps involved adding anhydrous sodium sulfite to salt-out the drug into the

extraction solvent. Using this method, about twenty other drugs were found not to interfere, and eight other common xanthines were resolved from both dyphylline and theophylline. Although theophylline was used as the internal standard it was claimed that β-hydroxypropyltheophylline would be a suitable alternative internal standard. About fourteen samples could be analyzed in 2.5 h.

3.2.3. Propoxyphylline

See *3.2.2. Dyphylline* for this drug, Wenk et al. [89].

3.2.4. Bamifylline

Another theophylline derivative, bamifylline, and three of its metabolites were assayed [92] by RP-HPLC on a 3-μm ODS column. Plasma (2 ml) was subjected to a series of extraction steps prior to HPLC assay over the concentration range 0.01—2 μg/ml. Fenetylline was the internal standard and the limit of detection for all compounds was found to be 0.01 μg/ml. No interference was observed with uric acid, creatinine, and caffeine or with twelve non-xanthine type drugs tested including the antiasthmatic drug, albuterol. At 0.5 and 1.0 μg/ml levels of spiked plasma, the recovery of bamifylline was 94% and 90%, respectively, while the various metabolites ranged from 62% to 75%. At 0.2—1.0 μg/ml levels C.V. and C.V.(R) were less than 8.2% and 6.3%, respectively. Sample stability remained after freezer storage for two months. This method was applied to subjects receiving drug (300 mg b.i.d.) for up to one month. The chromatographic run time was about 15 min; however, no indication of total sample preparation time was given nor were interferences due to many other common xanthines considered.

3.3. Anticholinergics

3.3.1. Atropine

Atropine has been used in medicine for a long time as an anesthesia premedication, as an antidote in pesticide poisoning, in the treatment of bradykardia hypotension syndrome in infants and adults, etc. As an antiasthmatic drug with bronchodilating properties, it has been successful in the treatment of chronic bronchitis and perennial childhood asthma. According to Kradjan et al. [93] the sulfate of atropine was inhaled as an aerosol at 0.05 mg/kg. Detectable levels of atropine in serum appeared within 15 min and all asthmatic patients had a good bronchodilator response with the peak blood levels ranging from 1 to 7.5 ng/ml. Only one subject had an exceptionally high peak of 21 ng/ml with attendant systemic side effects. The lowest detectable levels were reported to be 0.6 ng/ml. Atropine sulfate was also administered by other routes (i.v., s.c., i.m., p.o.). The plasma or serum levels ranged from a high of 83 ng/ml [94] to a low of 0.6 ng/ml [95]. Only partial information was available on the metabolism of atropine in man. Approximately one-half of an i.m. dose was excreted in the urine as unchanged drug and one-third as metabolites, probably esters of tropic acid [96]. The β-glucuronide was claimed to be a major urinary metabolite and some N-demethylation was also observed [97]. All of the above data on atropine levels in the circulation

were obtained by RIA or with radiolabeled drug. The lack of chromatographic methods suitable for DLM until very recent times is surprising.

The first chromatographic procedure for the determination of atropine in rat plasma (and brain) was published by Palmer et al. [98]. It was based on GC separation of the silyl derivative on 3% OV-225 on a Gas Chrom Q column at 170°C and MS quantitation using the ion peak *m/e* 124 (and *m/e* 127 for the deuterated drug as internal standard). No statistics for analysis of tissues were given and the recovery was 90—95%. The reported limit of detection of 1 ng/g of tissue homogenate would certainly warrant an adaptation of this assay to human plasma.

As part of a drug disposition study Eckert and Hinderling [99] described a sensitive GC—MS assay in the range 2—500 ng/ml in human plasma and 2—5000 ng/ml in urine [99]. Before chromatographic determination, atropine was hydrolyzed to tropine and derivatized with N-heptafluorobutyrylimidazole. This was injected onto a GC capillary column (15% GE SF-96 phase) at 200°C with helium as carrier gas and passed into a quadrupole MS system. The peaks *m/e* 124 and 127 (for internal standard d_3-atropine) were monitored. Because tropine was a suspected metabolite of atropine, a separation of drug and tropine preceded the hydrolysis step. A very complicated sample preparation procedure involving five chloroform extractions was used before derivatization. As could be expected, the recovery was low (45%), yet an amazing C.V. of 4.4% at 2 ng/ml was achieved in plasma. At the higher concentrations the C.V. was even less than 1%. A similarly low C.V. value was reported for urine, except for 29% at 2 ng/ml. The limit of detection was not given. Although atropine can be distinguished in this assay from metabolically formed tropine, no attempts were made to determine the latter in plasma and urine of a volunteer dosed with the drug. This assay would be too complex for routine DLM.

The use of GC with electron-capture detection for atropine determination in tissue was proposed by Green [100] in his preliminary communication. The range studied, 289—28.9 ng (amount of atropine base injected on column), was above the expected concentration range of drug in the circulation of man. No data on recovery from the biological matrix, C.V., accuracy, or limit of detection were presented. The same methodology was used in a drug screening assay study of 70 drugs, including atropine [101]. The limit of detection was 2.5 ng with no other evaluation of the assay discussed. Based on the listed retention times other commonly used drugs (propranolol, chlorpheniramine) would interfere. This assay could not be used in DLM.

HPLC has not been applied in DLM of atropine although fluorescence monitoring of derivatized drug could be a promising approach, as Wintersteiger [102] has shown with the corresponding anthracene—urethanes used for quantitation of alcoholic groups by TLC.

For use in DLM, only the GC—MS methods can compete with the sensitivity of the RIA-based assays [103—105], but the selectivity with regard to the presence of metabolites and other antiasthmatic drugs has not been studied. A radioreceptor assay for atropine in human plasma was also developed [106]. It was based on a simple procedure using the ability of antimuscarinic compounds to compete with tritiated quinuclidine benzilate for binding sites.

It has been applied to plasma level determinations of atropine in man after a 2-mg i.m. dose. The limit of detection was 0.9 ng/ml. Here again, the selectivity was also a problem.

3.3.2. Ipratropium bromide

This drug is an effective bronchodilator, administered by inhalation, and has a 4—6 h duration of action with very few systematic side-effects at prescribed doses. The recommended inhaled dosage for adults or children is 20—40 μg three times a day [107]. The plasma levels after p.o. administration of 30 mg reached 25 μg/ml at 3 h and remained high for an additional 3 h. When 555 μg was inhaled, a peak plasma concentration of 60 pg/ml was achieved at 3 h [108] and less than 10 pg/ml at 24 h. For the effective dose this level was much lower. Measurements of these extremely low drug plasma levels were accomplished with ^{14}C-labeled drug.

No chromatographic or other methods for DLM of ipratropium bromide in man have been published yet. We decided to mention this drug as a challenge to the bioanalytical chemist. Note that picogram levels of other analytes have been successfully determined, such as amino acids (with fluorescamine), melatonin in human plasma at 1—100 pg/ml [109], or tryptophan metabolites in brain and cerebrospinal fluid at 5—25 pg [110].

3.4. Corticosteroids

3.4.1. Cortisol

The use of certain corticosteroids in the treatment of asthma is generally limited to severe cases which are refractory to other therapy [111—115]. Choice of steroid and its dosing regimen and formulation (i.v., oral, or aerosol) vary with patient status [112—116]. The determination of these agents in biological fluids in asthma therapy is apparently not widespread. However, it is important in (a) assessing baseline steroid levels, (b) the return to normal adrenal cortical function following the withdrawal of corticosteroid therapy [111, 115], or (c) in assessing exogenous steroid elimination such as methylprednisolone which is reduced in the presence of troleandomycin (a common co-therapeutic agent for the steroid dependent asthmatic) [117, 118]. Research on the pharmacokinetics and pharmacodynamics of various corticosteroids is still being carried out [116, 119]. Normally, about 90% of cortisol is bound to plasma proteins (corticosteroid-binding globulin and albumin) and renal excretion is the major elimination route [111].

Some general considerations regarding cortisol are that solid-state extractions may not effect suitable clean-up as indicated by Schöneshöfer et al. [120]. Considerable UV-absorbing background interference was noted in 25% of the samples assayed by this HPLC UV-monitored serum cortisol assay in which cortisol and other androgen or progesterone steroids were first cleaned-up by Sep-Pak C_{18} cartridges [121] and the eluents then quantitated by RIA technique and HPLC.

Kawasaki et al. [122] attempted to overcome the interference problem using Dns-hydrazine derivatization of cortisol with subsequent fluorescence detection. Extraction of cortisol from plasma and urine used the liquid—liquid

technique. Although no internal standard was employed, plasma and urine cortisol recoveries were greater than 90% and C.V.(R) was 3—6%. The limit of detection was 1 ng/ml for plasma and 0.5 ng/ml for urine. In the case of the HPLC procedure described by Bouquet et al. [123] the use of equilenin as the internal standard for the cortisol and 11-desoxycortisol plasma HPLC method was criticized [124] because this internal standard contained a phenolic hydroxyl group which might be susceptible to the alkaline wash procedure employed in the extraction steps. In addition other corticosteroids such as triamcinolone, prednisone, and dexamethasone would interfere with the cortisol assay unless the mobile phase was altered as indicated in this paper.

Using HPLC with fluorometric detection, the very sensitive determination of cortisol in plasma [125] in the range 5—150 ng/ml was accomplished on normal-phase microparticulate silica using ethylene dichloride—butanol—water (19:8.5:0.5) as mobile phase. The C.V. was 3—12% with recovery calculated as 83% for cortisol. Dexamethasone did not interfere with cortisol, and this assay [125] was used to quantitate the cortisol levels in plasma from a limited number of normal volunteers. The sample extraction and Dns-hydrazine derivatization steps were lengthy (> 1 h) and, as stated, this method would be more applicable in determining endogenous cortisol levels.

Toothaker et al. [126] compared a modified version of the HPLC—UV detection procedure of Scott and Dixon [127] with the fluorescence detection method of Goehl et al. [125] and found them to give equivalent plasma cortisol levels (r = 0.987). The modified method used 1 ml of alkalinized plasma containing 200 ng of internal standard (Δ^4-pregnene-17-α-20β,21-triol-3,11-dione) and extraction with methylene chloride. The residue was chromatographed on an RP silica column using aqueous methanol as the mobile phase with UV monitoring at 254 nm. Results were corrected for endogenous cortisol over the concentration range 5—700 ng/ml. Replicate analyses gave a C.V. of 4—8% over this range with 82% cortisol and 83% internal standard recoveries. Both assays [125, 127] were used to determine pharmacokinetics in human subjects dosed with 10, 30, and 50 mg of cortisol. Regardless of the method employed, cortisol metabolites were claimed to be absent. Overall, the UV detection procedure [127] would be preferable because of its simplicity, speed in sample handling, and its comparable sensitivity even for cortisol-depressed plasma samples. The upper range of this method may not be suitable for bolus doses of cortisol administered for acute asthma.

Two more definitive HPLC methods [124, 128] are more thoroughly discussed because they used isotope dilution mass spectrometry (ID-MS) as a reference method for comparison of corticosteroid quantitation (see Table 5).

Lambert et al. [124] used two different internal standards, namely 19-nortestosterone and 6-α-methylprednisolone in their HPLC serum cortisol assay. Alkalinized samples were extracted in dichloromethane and the organic layer was washed with water. Chromatography employed an RP 5-μm C_{18} particle column with a mobile phase of methanol–water (ratio 50:50 or 57.5:42.5) and UV monitoring at 254 nm. Using 200 μl serum, the limit of detection for cortisol was 10 ng/ml, recovery 104%, and C.V.(R) 1.7% at the 90 ng/ml concentration level. Comparison of this HPLC method [124] with both RIA and fluorometry gave generally lower results which was attributed to inter-

TABLE 5

SUMMARY TABLE OF RECENT HPLC METHODS FOR VARIOUS GLUCOCORTICOSTEROIDS IN PLASMA OR SERUM

Analyte(s)	HPLC reference	Year published	Comparison method
Cortisol	124	1983	ID—MS
Cortisol	125	1979	None
Cortisol	126	1982	HPLC—fluorescence
Cortisol, prednisolone	128	1982	ID—MS
Cortisol, prednisolone, prednisone	130	1979	RIA
Cortisol, methylprednisolone, methylprednisolone hemisuccinate	131	1984	None
Methylprednisolone, methylprednisolone hemisuccinate, cortisol, cortisol hemisuccinate	132	1979	None
Prednisolone	143	1982	None

ference in the RIA and fluorometry methods. The HPLC method gave excellent correlation with the ID-MS assay results (90.1 versus 90.8 ng/ml, respectively). Samples were stored at 4°C and 20°C and analyzed up to three months later with no deterioration noted. Clinical samples, involving normal as well as elevated or depressed cortisol levels, were analyzed by these procedures. Other common endogenous or exogenous steroids did not interfere.

Oest et al. [128] simultaneously determined cortisol and prednisolone in serum by extraction and chromatography according to an earlier HPLC method [129], which used dichloromethane extraction followed by chromatography on 5-μm NO_2-substituted silica using dexamethasone as the internal standard and a mobile phase of ethanol—dichloromethane (3:97) with UV monitoring at 254 nm. The accuracy of the HPLC method when compared to the reference ID-MS method showed good correlation (prednisolone, r = 0.99 and cortisol, r = 0.95). The C.V. over the range 0—2200 nmol/l for both compounds was 2—3% for cortisol and 1—3% for prednisolone. The limit of detection was about 30 nmol/l for each compound with accuracy ranging from 1.4% to 2.4% for prednisolone and cortisol, respectively. The procedure was used to analyze over 500 patient samples and was claimed to be sufficiently accurate for monitoring the levels of cortisol during and after prednisolone therapy.

In 1979 Rose and Jusko [130] described an HPLC method and extraction procedure for simultaneously quantitating cortisol, prednisolone, and prednisone in plasma, urine, or saliva in the range 50—500 ng/ml at 254 nm by UV monitoring. For separation a 5-μm silica was used with a mobile phase of methanol—methylene chloride (3:97) and dexamethasone as the internal standard. Standards containing drugs and internal standard were prepared in cortisol-stripped (charcoal-absorbed) human plasma and 1-ml samples were extracted with methylene dichloride followed by sequential washing of the retained organic layer with sodium hydroxide and water. Recovery of cortisol and prednisone from plasma was 83%, the limit of detection was 5 ng/ml (signal-to-noise ratio 2.5), with a C.V. of 8%, 5%, and 12% for cortisol,

prednisolone, and prednisone, respectively. Selected corticosteroids and metabolites did not interfere. An unidentified RIA method gave comparable plasma levels to this HPLC method [130] (r = 0.985). The procedure was used in assaying over 2000 samples. Insufficient data were given to evaluate this method in saliva or urine.

Ebling et al. [131] used verbatim, the HPLC method and extraction procedure of Rose and Jusko [130] changing only the mobile phase to hexane—methylene chloride—ethanol—acetic acid (26:69:3.4:1) to quantitate cortisol, methylprednisone, and its hemisuccinate ester (E) in plasma. About 60% recovery was found for these drugs as well as for the internal standard, dexamethasone, at the 50 and 500 ng/ml levels. The C.V. and C.V.(R) at these levels were <5% except for E, which was quantitated by difference (hydrolyzed versus unhydrolyzed). Selected corticosteroids were found not to interfere, however, beclomethasone would probably interfere with dexamethasone and prednisolone with methylprednisone. Other common drugs and their metabolites, including theophylline and terbutaline, did not interfere. Repeated assays of patient samples after twelve months or longer of freezer storage gave identical steroid concentrations. This method would be more practical if automated and could possibly be used to quantitate dexamethasone if it could be recovered uniformly over a specified concentration range.

As two separate assays in this publication [132] either for methylprednisolone and its hemissuccinate ester (MPHS) or for cortisol and its hemissuccinate ester (CHS), all were quantitated using separate internal standards on RP 5-μm ODS stationary phase. Recoveries were: methyl prednisolone 80%, MPHS 94%, cortisol 80%, CHS, 85% over the ranges 0.1—2.95 μg/ml and 0.8—8.2 μg/ml, respectively. All assays required blank corrections for endogenous cortisol, which was calculated as 0.1 μg/ml. Analysis of thirteen standard curves over a three-week period gave C.V. > 0.99. The C.V.(R) values were all less than 5% and an estimation of the limit of detection was about 0.02 μg/ml for all analytes. The C.V. for each analyte over their respective concentration ranges in either serum or plasma were found to be 6.8% (methylprednisolone), 5.8% (MPHS), 2.9% (cortisol), and 6.1% (CHS), while accuracy was found (percentage differences) to be 3.3% (methylprednisolone), 4.8% (MPHS), 3.6% (cortisol), and 3.9% (CHS). This assay was applied to samples from two human subjects following i.m. administration of 125 mg methylprednisolone or 250 mg cortisol over a time course of 12 and 8 h, respectively. One drawback to this method, however, was that no studies were done on the possible interference due to other naturally present or co-administered steroids.

RIA methods for drug level monitoring for corticosteroids have found prominence in the literature mainly for cortisol in plasma [133—135], urine [136], and saliva [137, 138]. The common pitfalls of these cortisol assays were reviewed elsewhere [139].

3.4.2. Prednisolone

Prednisolone therapy to asthmatics is still largely empirical (10—60 mg per dose at 4—8 h intervals) by oral or i.v. routes. In general, rapid absorption is seen with a maximum concentration at about 1 h (600—1800 ng/ml) and a

plasma half-life of 30—250 min. Details of the non-linear pharmacokinetics and overall disposition in man may be found elsewhere [140, 141]. However, diurnal variation in prednisolone kinetics has been documented [142] which suggested that for maximum efficiency and lower toxicity, drug therapy should be confined to a single morning dosing regimen. Since inter-individual prednisolone blood levels vary widely, the recent literature suggested the measurement of free drug rather than total serum drug levels to give a more true dose—effect relationship [141].

HPLC assays for prednisolone [128, 130] and methylprednisolone [130, 132] in the presence of other steroids has been reviewed previously under *3.4.1. Cortisol.* A recent article [143] reported a plasma prednisolone HPLC procedure quantitating this drug alone over a range of 25—125 ng/ml. The separation of other steroids, prednisone, and cortisol was achieved but they were not quantitated.

3.4.3. Methylprednisolone

For methylprednisolone see *3.4.1. Cortisol.*

3.4.4. Dexamethasone

No practical new methods so far as asthma therapy DLM were found for this drug. However, two chromatographic procedures using the MS detection mode [144, 145] are only referred to. They did not provide sufficient data for evaluation, but they do point to possible future directions for selective and sensitive methodologies other than the currently available RIA techniques.

3.4.5. Triamcinolone

This drug has been reviewed elsewhere [146]. At therapeutic doses it gives peak serum levels (not specified) in 2—5 h with a half-life of 4 h. These low doses (about 0.5 mg range) coupled with the low nanogram levels achieved in biological fluids would probably require a sensitive method such as a RIA procedure [146]. A recent publication [147] gave a procedure for estimating triamcinolone in human urine by an on-line sample preparation mode coupled to HPLC. The automated sample preparation steps used a device developed by the authors (patent applied for) which concentrated the drug prior to chromatography. Assay evaluation was based on only one drug urine concentration level of 200 μg/l. The C.V. was 3.3%, C.V.(R) 5.6%, limit of detection 5 μg/l (signal-to-noise ratio of 3) with recovery 94%. No internal standard was used and the UV detection of drug was compromised whenever aged urine specimens were assayed or whenever high doses of antibiotics or glucocorticosteroids were present in patient specimens. Each assay required totally 40 min (48 samples per day). This procedure could have potential future DLM application for triamcinolone and other drugs in a variety of biological fluids.

3.5. Prophylactic agents

3.5.1. Ketotifen

This drug was effective in long-term prophylaxis of attacks in uncomplicated asthmatic adults or children when given orally twice a day at doses of 0.5—2

mg [148—150]. Sedation was the only side-effect. In man it is metabolized to the N-glucuronide (I), the 10-hydroxy (II), the pharmacologically active N-desmethyl (III), and the 10-hydroxy-N-desmethyl (IV) derivatives and the glucuronides of II and IV [151—153]. It was claimed that I was found in plasma in large amounts [154]. The therapeutic plasma drug level range has not been established yet but from the above references a low of 0.2 and a high of 15 ng/ml appears to be a reasonable estimate of this range.

A citation of a GC assay in urine was found in the literature [154] which referred to an internal report from Sandoz. Only one GC—MS method for the assay of this drug and its metabolites II, III, and IV in human plasma has been published recently by Julien-Larose et al. [154]. The free and conjugated drug and metabolites III and IV were determined in the range 0.05—1 ng/ml and the metabolite II in the range 0.3—8 ng/ml. The clean-up procedure for the drug and metabolite II consisted of an extraction of 1 ml plasma after addition of sodium hydroxide with benzene, centrifugation, and injection onto the capillary column. Metabolites III and IV were derivatized with HFBA before injection. Quantitation was based on the ion peak *m/e* 309 (drug) and *m/e* 293 (metabolite II). The internal standard was the drug analogue pizotifen, *m/e* 295. Similarly, for the N-desmethyl metabolites III and IV and their internal standard thioxanthen after derivatization the ions *m/e* 491, *m/e* 475, *m/e* 475 were monitored. The amount of conjugated drug and metabolites was determined after enzymatic hydrolysis. The lowest concentration in the range was declared to be the limit of detection. Excellent to acceptable C.V. values and accuracy were achieved for all concentrations. This assay was the only important contribution to the monitoring of ketotifen and metabolite levels in human plasma because it simultaneously determined four components with a relatively simple procedure. Other assays have, to our knowledge, not been published.

3.5.2. Disodium cromoglycate

Two reviews on the disodium cromoglycate (DSCG) pharmacology and mechanisms of action [155] and on general properties and clinical use [156] have been recently published. DSCG is an effective long-term treatment for a wide variety of bronchial disorders including asthma. The exclusive route of administration is by inhalation. The recommended dose is 20 mg four times a day. Very little of an oral dose is absorbed, but approximately 8% of the inhaled dose reaches the lung. Plasma levels in asthmatic adults ranged from 6.5 to 12.1 ng/ml at 15 min to about 2 ng/ml at 3 h [157], as determined with radiolabeled drug. In asthmatic preschool children at 10 min after inhalation, most patients showed serum levels less than 10 ng/ml, but a high of 64 ng/ml was also recorded [158]. In man this drug is not metabolized [157, 158].

A fluorometric assay, developed by Moss et al. [159], was first used to determine drug plasma concentrations in volunteers after receiving higher than therapeutic doses of DSCG. This method has a limit of detection of 100 ng/ml and was not suitable for DLM.

In 1979 a simple HPLC procedure for the determination of drug in human urine was published by Tomlinson et al. [160]. It consisted of direct injection of 1 μl of urine onto an RP C_{18}-Spherisorb ODS column with a methanol—

water (1:1) mobile phase containing decylbenzyldimethylammonium chloride as ion-pairing reagent and aniline hydrochloride as the internal standard. The limit of detection was 0.33 μg/ml. Considering the excretion of 2% of the dose in urine, this limit of detection should be sufficient for monitoring patient urine after a 20-mg dose four times a day (estimated level of 3 μg/ml in 24-h urine). No statistical evaluation of this assay was presented, but the paper had a valuable detailed discussion of ion-pair retention mechanisms in order to optimize the separation.

A lower limit of detection (0.05 μg/ml) was achieved in Gardner's HPLC assay [161] by introducing a double extraction step (two extractions with diethyl ether, back-extraction with a glycine buffer). Chromatography involved separation on an ion-exchange Partisil SAX column with a phosphate buffer (pH 2.3) mobile phase and UV detection at 325 nm. The drug recovery for the concentration range 0.2—20 μg/ml was found to vary from 66.7% to 79.7%. The C.V. was better than 6% and the accuracy varied from 0.5% to 11.8%. This assay was a significant contribution by also addressing the areas of interference and automation (40 samples per two days). Theophylline, terbutaline, prednisone, cortisol, and four other drugs did not interfere, however, their metabolites, which were also present in the urine in large amounts, were not considered.

Presently, only a RIA method is available for the determination of drug levels in human plasma [162]. It used sheep antiserum and a ^{125}I heterogenously labeled radioligand. The C.V. was less than 14% for concentration between 0.46 and 70 ng/ml, which covered the concentration range found in human plasma up to 8 h post-dosing. The cross-reactivity with other antiasthmatic drugs (prednisone and theophylline) was low. The advantage of this procedure was the small amount of plasma (10—100 μl) needed.

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5. SUMMARY

In general assays pertaining to drug level monitoring (DLM) of antiasthmatic agents (except theophylline), published during the period 1978—1983, used mostly high-performance liquid chromatographic (HPLC) methodology (approximately 45%) with mass spectrometric (MS) based assays in second place (approximately 30%) followed by immunochemical techniques (approximately 25%). Whenever nanogram or subnanogram antiasthmatic drug concentrations had to be measured such as for the adrenergic stimulants or for the prophylactic agents, then both HPLC- and MS-based methodologies were employed with about equal frequency.

The trend in DLM for the phosphodiesterase inhibitor class (theophyllines) seemed to be shifting towards the HPLC methodologies. In part, this was justified by the need for improved selectivity. This criterion appears to have been better satisfied by HPLC, but for all practical purposes the immunochemical methods are and will probably continue to prevail in the clinical laboratory setting until HPLC procedures become truly automated.

In the case of DLM of corticosteroids used for the asthmatic, the situation is in our opinion still unclear. This is caused by the presence of endogenous corticosteroids and metabolites, the levels of which in man are known to vary. The current immunochemical procedures offer a facile but less selective option. The future for selective routine corticosteroid assays may well be in HPLC or gas chromatography coupled with MS.

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