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Determination of methotrexate and indomethacin in urine using SPE-LC-DAD after derivatization

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ABSTRACT

A validated HPLC-DAD assay is presented for the simultaneous quantification of methotrexate and indomethacin in a drug combination which is used synergistically to intervene with tumoral or inflammatory tissue microenvironments. The analytes were isolated from urine via solid phase extraction. The method is based on derivatizing both analytes with a soluble carbodiimide coupler and 2-nitrophenylhydrazine directed to their commonly occurring carboxylate functions. The chromatographic separation was accomplished on an octylsilica column in less than 15 min using acetate buffer (pH 4; 10 mM)–methanol (60:40, v/v) as eluent at 1.5 ml/min and monitored at 400 nm. The selectivity of the method was demonstrated in a pre-dosed rheumatoid arthritis patient. Quality control samples were prepared and analyzed to reveal the validity of the method. Life samples collected from a healthy volunteer were monitored for both drugs and their urinary levels were determined.

1. Introduction

Methotrexate (MTX, amethopterin) (Fig. 1) is a folate-antagonist exhibiting pronounced antiproliferative activity. In high doses, MTX has for decades been in clinical use as a cytotoxic drug for solid neoplasms and leukemias [1]. It has also been used in low doses for the treatment of some autoimmune and inflammatory diseases such as psoriasis and severe steroid-dependent asthma, and as a disease modifying agent in rheumatoid arthritis [2].

Non-steroidal anti-inflammatory drugs (NSAIDs) suppress pain and inflammation in rheumatoid and psoriatic arthritis by inhibiting cyclooxygenases-mediated prostaglandin synthesis. Recently, some NSAIDs have been implicated in cancer chemotherapy and chemoprevention. In addition to the anti-tumor activity of NSAIDs as single agents [3], there is interest in the effects of combination chemotherapy with NSAIDs. For instance, MTX combined with indomethacin (INDO) (Fig. 1) showed, in experimental animals, synergistic anticancer potential in neuroendocrine carcinoma [4]. A similar finding was noticeable in humans with recurrent small cell lung carcinoma refractory to intensive conventional chemotherapy using cisplatin, etoposide, and cyclophosphamide [5]. Besides, the co-administration of MTX and NSAIDs is a common practice in cancer and rheumatoid arthritis in order to manage pain and inflammation. However, these combinations interact pharmacokinetically augmenting MTX-related adverse effects [6]. Being anions, MTX and most NSAIDs are excreted into urine via organic anion transporters (OATs) [7]. Competition for OATs is probably the most important mechanism underlying the interaction between MTX and NSAIDs in humans [8,9]. Furthermore, MTX and NSAIDs are both highly protein bound drugs (~50% and ~90% respectively); therefore, they are likely also to interact by displacing each other from plasma proteins [9]. The majority of MTX dose is excreted unchanged in urine, whereas smaller but significant amounts of NSAIDs do [10,11]. It is noteworthy that alkalinizing agents such as sodium bicarbonate (infusions or capsules) are frequently given with MTX in order to maintain its solubility by shifting the urinary pH >7.0, and thus avoiding its precipitation and minimizing its nephrotoxicity. Consequently, the excretion rate of NSAIDs is expected to increase when concomitantly administered with these urinary alkalizers. Taken together the aforementioned data suggest that the interactions between MTX and NSAIDs might result in unpredictable urinary excretion of the unbound form of both drugs. Therefore, monitoring urinary levels of binary mixtures of MTX and NSAIDs is of paramount importance.

Concerning the quantification of MTX and its metabolites or structural analogues in biological fluids liquid chromatography predominates. This has been intensively reviewed by Rubino [12]. In general, the analytical literature and pharmacopoeias are

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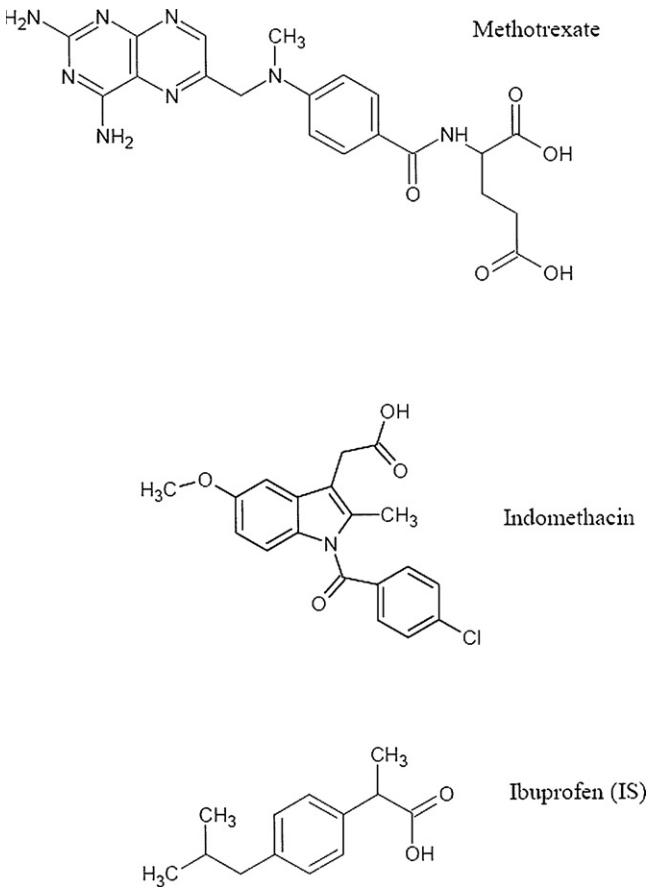


Fig. 1. Chemical structures and generic names of the studied compounds and the IS.

overwhelmed with various methods for the determination of INDO and other NSAIDs in different matrices [13–18]; however, none is appropriate for their simultaneous assay with MTX in biological fluids. The objective of the present work is to develop an analytical method to determine MTX and NSAIDs in human urine with a single chromatographic run after a solid phase extraction and clean-up step. We aimed at chemically modifying the common carboxylic functionalities in these chemically divergent entities, MTX and INDO. Derivatization adds to the selectivity and sensitivity of the method and thus, enables monitoring MTX in low-dose therapies as in rheumatoid arthritis without interference from co-administered structurally related analogues, such as leucovorin (LV) or folic acid (FA) [19]. The proposed assay method could be straightforwardly extended to determine other NSAIDs such as ibuprofen (IBU) (Fig. 1), the internal standard (IS) in our method, and diclofenac sodium or potassium (DIC) alongside with MTX in biomatrices.

2. Experimental

2.1. Chemicals and reagents

MTX in its base form and LV as its calcium salt pentahydrate, of purity 99.0% and 99.6% respectively, were purchased from PIChemicals, Shanghai, China. INDO (99.5%), IBU (99.4%), DIC sodium (99.2%), and FA (99.2%) were kindly donated by local pharmaceutical companies (Pharco and European Egyptian Pharmaceuticals, Alexandria, Egypt). Stock solutions of analytes in alkaline methanol, protected from light by aluminum foil, were kept refrigerated at ~4 °C. With respect to these storage conditions, these solutions were stable for about 2 months. A 2-nitrophenylhydrazine (2-NPH) (Fluka Chemicals, Buchs, Switzerland) solution (0.02 M) was prepared by dissolving the reagent in water containing 40% HCl (1 M)–ethanol (1:1, v/v). A N-(3 dimethylaminopropyl)-N′-ethyl-carbodiimide hydrochloride (EDC.HCl) (Fluka Chemicals, Buchs, Switzerland) solution (0.25 M) was prepared by dissolving the reagent in ethanol containing 3% pyridine. A 10% NaOH (Fluka Chemicals) solution was prepared by dissolving the pellets in water–methanol (1:1, v/v). Acetate buffer (pH 4; 10 mM) was prepared by dissolving the proper weight of sodium acetate (Merck, Darmstadt, Germany) in water and adjusting to pH 4 ± 0.2 with glacial acetic acid. All reagent solutions were stable for at least 3 months when kept at 4 ± 1 °C. All solvents used were HPLC grade (Lichrosolv Merck, Darmstadt, Germany). Doubly distilled water was used throughout the work. All chemicals used otherwise were of analytical grade.

2.2. Instrumentation and HPLC analysis

Chromatographic analysis was carried out using an HPLC-DAD system consisting of Agilent 1200 series (quaternary pump, vacuum degasser and diode array and multiple wavelength detector G1315 C/D and G1365 C/D) connected to a computer loaded with Agilent ChemStation Software. Sample injections were made through a Rheodyne manual injector with a sample loop size of 20 μl. The separation was performed throughout the experiments on a Zorbax SB-C8 column 5 μm, 250 mm × 4.6 mm I.D. (Agilent, California, USA). The corresponding guard columns SB-C8, 5 μm, 4 mm × 4.6 mm manufactured by Agilent, were replaced after ~100 injections. All analyses were carried out isocratically with a mobile phase consisting of acetate buffer (pH 4; 10 mM)–methanol (60:40, v/v) at a flow-rate of 1.5 ml/min. The mobile phase was

freshly prepared weekly, membrane-filtered and degassed prior to circulating into the system. The UV-DAD detector was set at 400 nm.

2.3. Calibration standards

Stock solutions of MTX and INDO were prepared by dissolving 50 mg of each compound in 50 ml of NaOH (0.1 M)–methanol (1:9, v/v). The IS solution was prepared by dissolving 25 mg of IBU in 50 ml of the same solvent. Working solutions of each analyte at different concentration levels were freshly prepared by appropriate dilution with the previous solvent mixture in amber-glass vials. Aliquots of these working solutions together with 15 μl IS were added to blank human urine prior to solid phase extraction (SPE) to prepare calibration standards resulting in 0.1, 0.4, 1.0, 2.0, 4.0, 8.0 and 10 μg/ml.

2.4. Extraction from urine

Blank human urine samples were allowed to thaw at room temperature, thoroughly vortexed for 10–15 s and aliquots of 1.0 ml urine, to which added 15 μl IS, were spiked with both analytes at the given concentration levels. Afterwards, 300–350 μl of acetate buffer (pH 4; 0.1 M) were added. To extract the analytes from the biofluid, Bond Elut® C18 100 mg/1 ml (Varian, Switzerland) cartridges were used. Just after conditioning the SPE system with 2× 3 ml of methanol and acetate buffer (pH 4; 0.1 M) respectively, 1.0 ml of the spiked urine-buffer mixture was loaded onto the SPE cartridge. Under gentle vacuum, the liquid was gently aspirated through the cartridge, and then the cartridges were washed twice with 1 ml of acetate buffer (pH 4; 0.1 M) and evaporated in vacuum nearly to dryness. Finally, the columns were eluted with 2× 0.5 ml of methanol. Parallel sets of human urine from 3 different sources without the active analyte (blank) were likewise processed. Eluted fractions were analyzed for their content according to the procedure in the following section.

2.5. Procedure

Eluates of the processed samples were quantitatively transferred into 2 ml calibrated volumetric flasks, and their corresponding recipient vials were washed twice with 100 μl methanol. Washings were similarly transferred, and 200 μl of EDC.HCl (0.25 M) followed by 300 μl of 2-NPH (0.02 M) were added. The mixture is vortexed for 20–30 s then heated at 80 °C for 3 min. After cooling, 200 μl of 10% methanolic NaOH solution is added and the reaction mixture is heated again at 80 °C for 3 min. The solution is then made up to its final volume with water. Finally, the reaction mixture is vortexed for 20–30 s and an aliquot of 20 μl is injected into the chromatograph.

2.6. Validation and control sample solutions

2.6.1. Validation standards

A new stock solution of each analyte was prepared at the same concentration as above. Validation standards were prepared in human urine containing 15 μl IS so as to result, after processing, in the following concentrations: 0.1, 0.4, 2.0, 8.0, and 10 μg/ml.

2.6.2. Control samples

2.6.2.1. Quality control samples. Three replicate sets of quality control (QC) samples were prepared by spiking 1.0 ml of blank human urine, from 3 different sources with 15 μl IS and aliquots of MTX and INDO solutions, not exceeding 5% of the matrix volume to yield 0.2 (low QC = 2–5 × LOQ), 2.0 (middle QC) and 10 (high QC) μg/ml. These samples were stored in polypropylene vials at −70 ± 3 °C until the day of extraction. In each sequence, QC samples of the aforementioned concentrations were analyzed in triplicate. The results of these QC samples provided the basis for accepting or rejecting the individual run. At least 6 out of 9 of the QCs had to be within ±15% of their respective nominal values. Up to 2 of the QCs could fail this criterion, unless they were prepared at the same concentration level. Analytical runs not meeting this criterion were repeated along with recording the reason of failure whenever possible [20].

2.6.2.2. Over-curve dilution control samples. Over-curve dilution samples were prepared by spiking blank human urine containing 30 μl IS in triplicate as above to yield a concentration of 20 μg/ml for each analyte. These samples were processed and the extracts were two fold diluted with extracted blank urine prior to analysis. Bias should not be compromised by comparison to the upper QC. Intra- and inter-day precision should as well conform to the regulatory guidelines [21].

2.7. Collection of real samples

A human male volunteer aged 34 who had normal blood chemistry and was in good health as reflected by medical history and examination by a physician was administered 50 mg MTX sodium (Methocip® 50 mg/2 ml vial, Cipla LTD. Verna Industrial Estate, Goa, India) by intramuscular injection, and 50 mg INDO (Indomethacin® 50 mg per capsule, Pharco Pharmaceuticals, Alexandria, Egypt) per os. The urine was *in vivo* alkalinized by administration of 1 g sodium bicarbonate in a capsule form to guarantee that urinary pH does not fall below ~7.0. Urine was collected before administering the drugs and for the next 10 h, each 2 h interval, into amber glass 1-l bottles containing 5 g of dry ascorbic acid; the bottles were kept refrigerated (~4 °C) during the collection period. Adequate hydration and normal diet were maintained throughout the collection period and urine pH was monitored. Immediately after the 10 h collection was completed, the volume of each urine sample was measured, and 5 ml portions were transferred to polyethylene vials, saturated with nitrogen gas, and stored at −70 ± 3 °C until analyzed. A 1.0 ml volume of each collected sample was extracted and analyzed on the same day according to the previous sections.

3. Results and discussion

3.1. Optimization of the derivatization conditions

Most NSAIDs show maximum UV absorbances at wavelengths below those of MTX (300–320 nm) in the designated mobile phase (pH 4–5) [22]. The pharmacopoeial HPLC assays of NSAIDs, including INDO [17,18] principally employ the universal but much less specific detection wavelength of 254 nm.

The chromatographic behaviour of underivatized MTX and NSAIDs vary tremendously; the former being a rather complicated polyelectrolyte molecule with a heteroaromatic pterine chromophore, whereas the latter group comprises simpler aromatic molecules that mostly possess an acidic functionality. Our preliminary experiments using direct isocratic separation were only successful in urine-free samples; however, a relatively long run time of > 25 min was required. Moving to biological samples, we faced troublesome separation mainly due to matrix interferences. Therefore, our next trial was devoted to pre-column derivatization which offered an appealing approach for the following reasons:

1. It shortened the runs significantly.

2. It provided adequate sensitivity for the quantification of NSAIDs because the amounts excreted unchanged of these are relatively smaller in comparison with MTX.
3. It enabled facile separation of folic and folinic acids from MTX, and thus, it noticeably added to the selectivity of the method in biological fluids.
4. It permitted chromatographing diclofenac because the derivatives were of comparable chromatographic performances.
5. It allowed internal standardization using ibuprofen which is not structurally related to MTX. It is noteworthy that IS selection is a matter of controversy in MTX assays [12].

Apart from a few modifications, we adopted a derivatization reaction originally described by Miwa [23] which transforms organic acids into the corresponding colored acyl hydrazides (Scheme 1). Derivatized carboxylic acids could be either monitored in acidic or alkaline medium. For INDO as a model, the derivative's absorption spectrum shows maxima at 402 nm and 534 nm at pH 1 and 11, respectively. On the other hand, derivatization of MTX resulted in a strong peak at 404 nm as well as 2 other maxima of lower intensities at 541 and 556 nm. These derivatives also show strong absorption in the UV region, with maximum absorption at around 230 nm. However, an excess of the reagents and the reaction by-products do interfere with the HPLC analyses at this wavelength. In contrast, most of them do not absorb visible light above 400 nm. Accordingly, the resultant chromatograms are simpler and more selective, in spite of a bit compromised sensitivity. For the studied analytes as well as the IS, a vivid violet color is indicative of a positive reaction.

In order to ensure the maximum derivatization of the carboxylic acids, the reaction conditions were briefly explored. Alkaline methanolic solutions of the investigated analytes react quickly with 2-NPH.HCl using EDC.HCl as a water soluble coupler to yield acid hydrazides [24]. The percentage of organic solvent in the reaction mixture is critical for the feasibility of the derivatization reaction. Best results were obtained using a ratio of approximately 5:1 between the organic and the aqueous phase, respectively. Acetonitrile and methanol were tested and the latter was the solvent of choice. Furthermore, the addition of aqueous alkali is important to diminish the blank reading. Temperature is a critical factor which controls the rate of acyl hydrazide formation. Although the derivatization rate steadily increases with rising temperature, the produced derivatives are liable to slight progressive degradation. Hence, an optimum reaction time of 3 min at 80 °C was found adequate to convert the analytes into their hydrazides. This conversion was due to the intact derivatives as demonstrated by quantitative and reproducible responses. Furthermore, different volumes of the coupling agent and the reagent were tested, and 200 μl of EDC.HCl (0.25 M) at 3% pyridine and 300 μl of 2-NPH (0.02 M) were optimal volumes. While 200 μl of the hydrazine reagent were sufficient to derivatize the analytes at the studied concentration levels, a slight excess was used in the biomatrix to account for physiologically occurring carboxylic acids and carbonyl compounds.

3.2. Optimization of the extraction and clean-up procedures

We used C18 cartridges which retained the analytes via apolar interactions. Anion-exchange cartridges (SAX) and mixed-mode anion-exchange/reversed-phase cartridges (MAX) were found to be inferior to C18 SPE columns in terms of extraction yield. A possible explanation for NSAIDs is the prevalence of their lipophilic character (log *P*(octanol/water) for INDO, IBU and DIC = 3.60, 4.00 and 3.90 respectively). However, the good retention of MTX on octadecyl silica columns could be interpreted by strong Van der Waals forces between the flat areas in this molecule and the sorbent molecules yet hydrogen bonding cannot be excluded. A volume not less than 300 μl of acetate buffer (pH 4; 0.1 M) in the loaded solution is essential in order to reduce the ionization of the analytes as much as possible, and hence enhance their retention on the extraction column. Washing with the previous buffer was reasonably sufficient to yield clean extracts, and consequently no further trials were implemented using escalating increments of organic solvents in the washing solution. To elute the compounds of interest, we tried alkalinized methanol (brought to pH 9–10 using ammonia) and methanol only as the extractor liquids. Both showed comparable elution efficiency, hence methanol was selected for the sake of simplicity. During the developmental and validation phases, exhausted columns were effectively used up to 5 times. However, such practise is not recommended during the routine use of the method.

3.3. Chromatographic separation

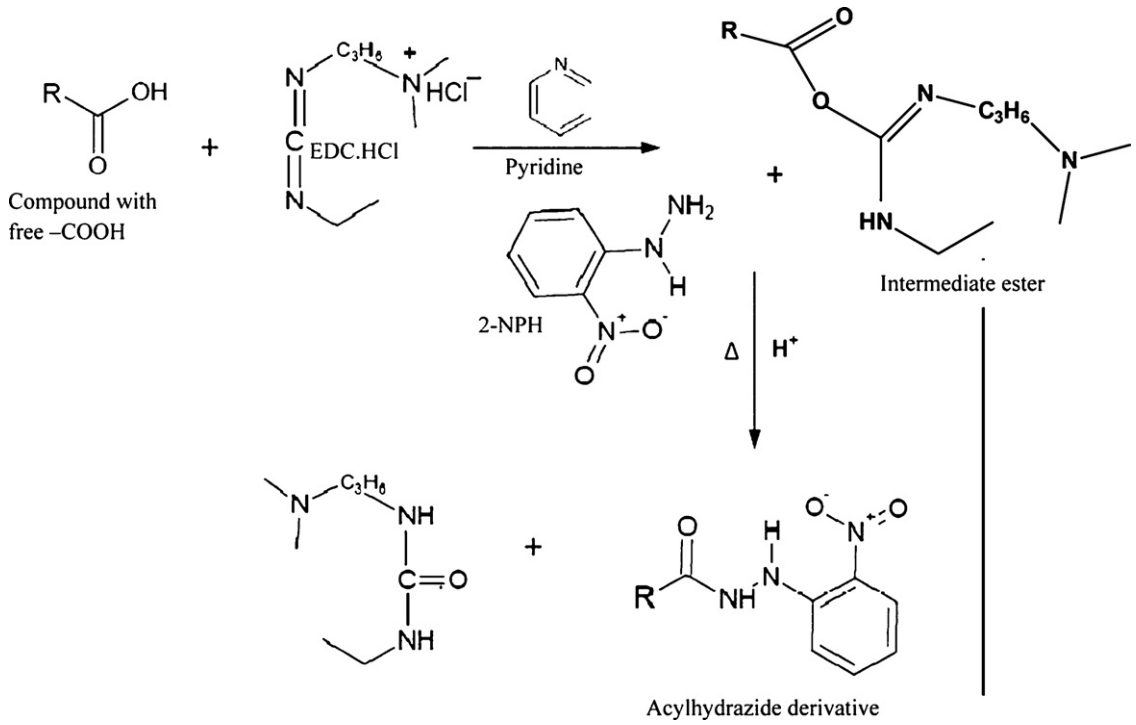
Two analytical columns and various mobile phase compositions were tried in order to reach an acceptable separation as well as a reasonable chromatographic run time. A C18 column was highly retentive for the analytes, and thus resulted in late eluting peaks especially for MTX (t_R 24 min). In contrast, base line chromatographic separation of the target analytes and the IS was accomplished on an octyl-silica column of intermediate hydrophobicity in 14 min under the described elution conditions (Fig. 2). As anticipated with nitrophenylhydrazine reagents, being a diacid MTX generated 2 peaks representing the mono- and di-carboxylic derivatives (t_R 8.7 and 13.2 min respectively). We depended on the mono-substituted derivative since it is the one which yielded a higher response that was of adequate sensitivity. However, both peaks can be used to quantify MTX. On the other hand, Indo showed 1 peak at t_R 3.5 min.

At first, we investigated an unbuffered eluent consisting of acidified water (brought to pH 4–5 using conc. HCl)–acetonitrile (50:50, v/v) but peaks were eluted early and were seriously disturbed by background signals. Addition of 20% methanol instead of acetonitrile did not improve the separation. Then, we tried a buffered mobile phase composed of acetate buffer pH 4 and methanol. Fine modulation of the previous composition resulted in an optimum mobile phase of acetate buffer (pH 4; 10 mM)–methanol (60:40, v/v) pumped at 1.5 ml/min. Detection was at 400 nm as reported by Miwa [23].

3.4. Validation

3.4.1. Calibration and linearity

Calibration curves were constructed by derivatizing increasing amounts of both MTX and INDO using EDC.HCl and 2-NPH and analyzing as previously described. The calibration test based on internal standardization was replicated 3 times. From the chromatograms obtained, the ratios of peak areas for the analytes to the IS were computed and the concentrations of both analytes were calculated by the least-squares method. Estimation of the response function was carried out by linear regression analysis, which was computed by the least squares fits with straight lines not forced through the origin. Being the simplest algorithm, this model was first verified in accordance with the Washington Conference Report. The quality of fit was primarily evaluated by comparing the calculated concentrations with their respective nominal values. The relative deviation of standards back-calculated from their nominal values did not exceed 7%; therefore, fulfilling the guidelines of bioanalytical method validation suggested in the literature [20]. The coefficient of correlation (r^2) was above 0.998 in each case; however, one cannot rely on the determination coefficients alone to validate calibration models. Thus, we further checked the robustness of the selected calibration model to fit the data by visually analyzing their residual plots (*y*-residuals versus the concentration), these



Scheme 1. Proposed mechanism of carboxylic acid activation with subsequent addition of the hydrazine reagent giving rise to the corresponding 2-nitrophenylhydrazide after dehydration.

showed randomly and symmetrically scattered residuals that fall within a constant horizontal band (homoskedastic) whose mean is zero indicating that the response was linearly and correctly regressed over the whole range of fitted values.

3.4.2. Limits of quantification/detection and range

LOQ and LOD, estimated on a basis of S/N ratio of 10 and 3 respectively (noise calculated peak to peak on a blank chromatogram at the analytes' t_R), were 0.08 and 0.03 μg/ml and 0.04 and 0.01 μg/ml for MTX and INDO, respectively for a urine volume of 1.0 ml. LOQs were validated by confirming their precisions and biases (via comparison with the lower QC in Table 1) using LOQ QC samples spiked with each analyte in triplicates. As a result, the linear dynamic range for the present assay method could be defined as 0.08- and 0.04–10 μg/ml for MTX and INDO, respectively.

3.4.3. Selectivity

The selectivity of a bioanalytical method regarding endogenous interferences is generally demonstrated by processing QCs in multiple matrix sources. Expectantly, considerable interferant impact would be perceived at low analyte concentrations, thus it is necessary to carefully examine the chromatograms, across the time windows of peaks of interest, at the individual

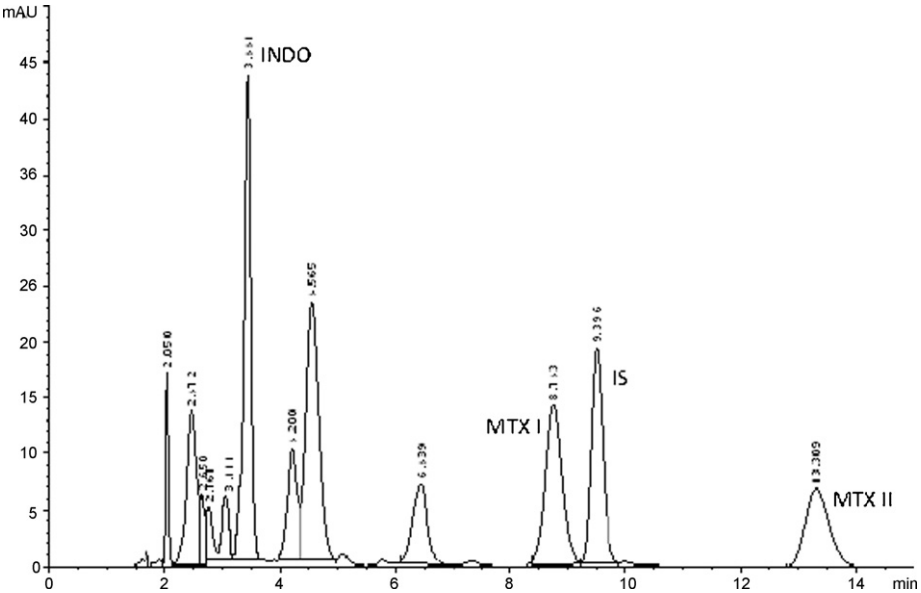


Fig. 2. Validated liquid chromatogram of human urine spiked with MTX (8 μg mL^{-1}) and INDO (2 μg mL^{-1}) processed and derivatized according to the conditions stated in the text (*I* and *II* stand for the *mono-* and *di-substituted* derivatives, respectively).

Table 1
Validation of MTX and INDO determination in human urine.

Trueness ($r = 3$, $n = 3$)[a] ($\mu g\,ml^{-1}$)	Absolute {relative} bias (MTX/INDO) ($\mu g\,ml^{-1}${%})	
0.2	−0.008 {−4.0}/−0.003 {−1.5}	
2.0	0.053 {2.7}/−0.007 {−0.4}	
10.0	−0.595 {−6.0}/0.473 {4.7}	
Precision ($r = 3$, $n = 3$) ($\mu g\,ml^{-1}$)	**Repeatability (MTX/INDO) (RSD%)**	**Intermediate precision (MTX/INDO) (RSD%)**
0.2	2.7/3.6	3.9/5.0
2.0	2.0/1.6	3.5/1.9
10.0	2.1/1.6	2.7/1.8

[a] r = runs and n = no. of replicates.

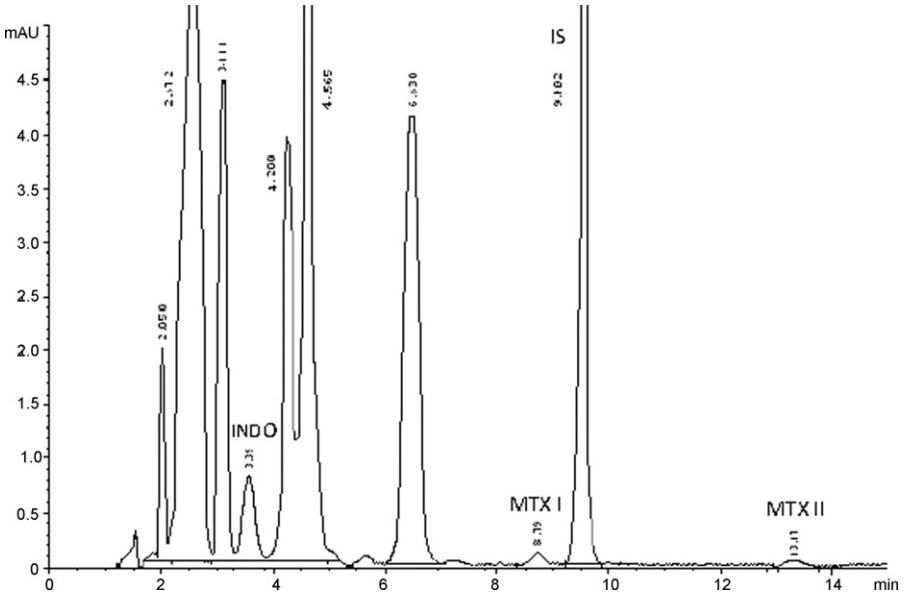


Fig. 3. Liquid chromatogram for a blank human urine spiked with MTX and INDO at the individual LOQs, processed and derivatized according to the conditions stated in the text (*I* and *II* stand for the *mono-* and *di-substituted* derivatives, respectively).

LOQs. Fig. 3 shows a typical LOQ chromatogram obtained after extracting a human urine pool spiked with MTX and INDO, at the individual LOQs, followed by derivatization. The absence of interfering signals at the retention times of both analytes in blank urine chromatograms is illustrated in Fig. 4.

Although structurally related pterins, viz, LV and FA reacted with 2-NPH in the presence of EDC.HCl, they did not interfere with

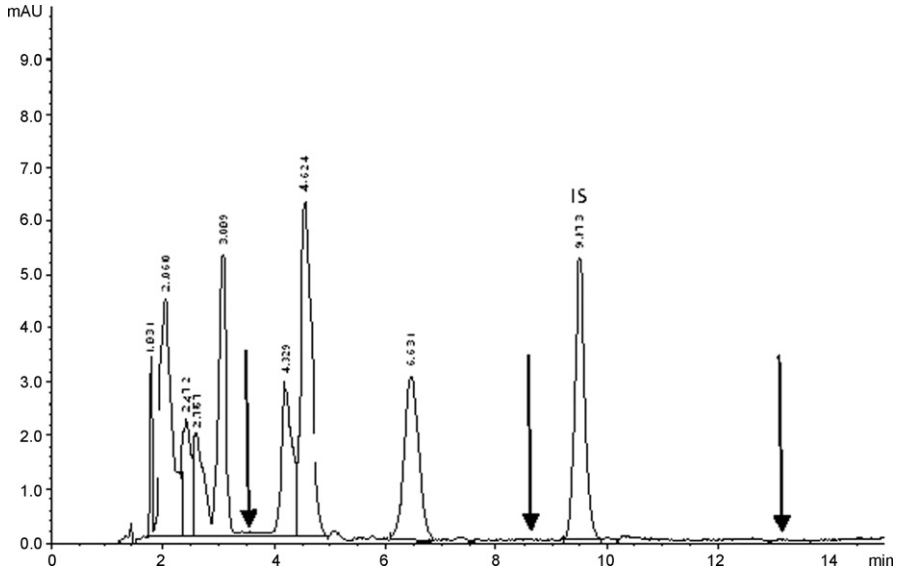


Fig. 4. Liquid chromatogram of blank human urine processed and derivatized according to the stated conditions within the text (the arrows indicate the t_R of analyte derivatives and IS).

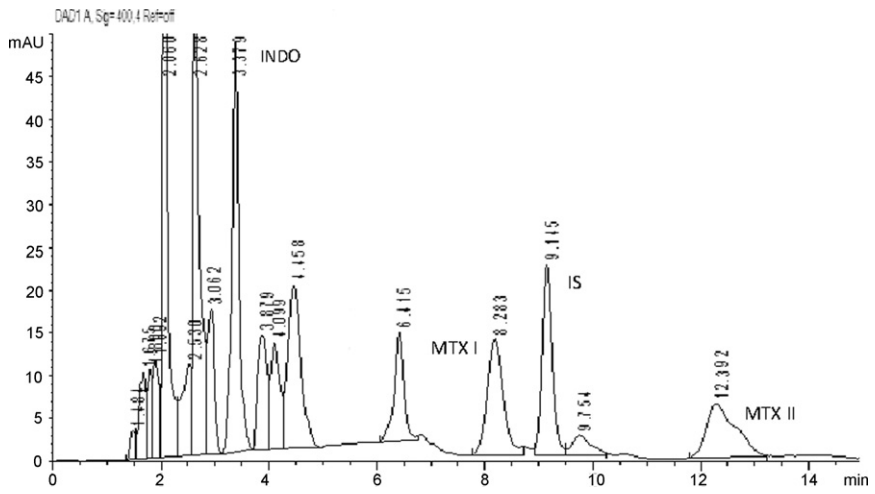


Fig. 5. A validated liquid chromatogram showing a real urine sample 2 h after administration of the studied drugs processed and derivatized according to the conditions stated in the text.

MTX peak. FA resulted in acid hydrazides which exhibited comparably eluting peaks at 7.6 and 12.1 min. However intriguingly, LV derivatives showed 5 peaks of substantially different polarities (t_R: 5.7, 7.0, 10.5, 16.1, and 29.4 min). These most probably represent mixtures of the hydrazones, monocarboxylic, and dicarboxylic acid hydrazides. Nevertheless, further spectroscopic experiments are required to elucidate their structures, a task which is beyond the scope of the current work.

To exclude natively occurring interferences in pre-dose samples, blank urine collected from a RA patient (on piroxicam, 20 mg/day; hydroxychloroquine 400 mg/day; and methylprednisolone 8 mg/day) was spiked with both MTX and INDO and chromatographically screened under 'stretched' conditions. Tolerable interferences, not hindering adequate peak integration, were detected (data not shown). Such interference was considered acceptable insofar as the bias of quantification at the LOQ was not compromised.

3.4.4. Extraction efficiency

The extraction recoveries of MTX and INDO from human urine were determined by comparing the absolute peak areas of samples spiked with the analytes prior to processing at 3 different concentrations, corresponding to the lower limit, middle point and upper limit of each calibration curve (i.e. urine levels of 0.2, 2.0 and 10 μg/ml) to those of drug-free control urine samples fortified post extraction with the same theoretical concentrations. The mean recoveries for MTX and INDO were calculated as 84% and 93% respectively. The repeatability of the recoveries at each concentration level was satisfactory as confirmed by an acceptable RSD% value which did not exceed 2.17 and 3.03, respectively ($n = 5$).

Table 2
Amounts of drugs excreted in real urine over 10 h.

Time (h)	mg excreted	
	MTX	INDO
0	–	–
2	15.3	4.2
4	7.8	1.1
6	6.3	0.5
8	4.1	0.2
10	3.2	0.2
Total (% dose)	73.4	12.3

3.4.5. Trueness and precision

Results of trueness expressed in terms of absolute bias (μg/ml) or relative bias (%), repeatability (intra-day precision), and time-different intermediate precision obtained from validation standards are summarized in Table 1. At the studied concentration levels, the within-run and between-run RSDs did not outstrip 5%. The overall bias never exceeded 6%. All values were within the limits of Washington guiding principles recommended for bioanalytical method validation.

3.4.6. Over-curve dilution control

Over-curve diluted human urine samples achieved biases and precisions comparable to those of the high QC (Table 1). Therefore, one can conclude that dilution of concentrations above the calibration curve to within range is tolerable in the routine usage of the method.

3.4.7. Stabilities

3.4.7.1. Storage and handling stability. The stability of MTX and INDO was tested under the conditions recommended by the FDA which are freeze and thaw stability, short-term temperature stability, long term stability, processed samples stability and stock solution stability [25]. The first three types were assessed with 3 sets of stability control samples (each set consisted of two concentration levels; low and high) after 3 freeze–thaw cycles, after storage at room temperature for 24 h and during storage at -70 ± 3 °C up to 30 days. The measured mean concentrations after freeze–thaw cycles and at room temperature or after storage below −60 °C did not differ significantly from nominal values. Further, the stability of both analytes in processed samples was determined after storage at room temperature or at 4 ± 1° C for 72 h. Stock solutions' stability was also tested after storage for 6 h at room temperature and after storage at 4 ± 1 °C for 2 months by comparing the instrument response with that of freshly prepared solutions. It was concluded that MTX and INDO are stable under these circumstances.

3.4.7.2. Bench-top and derivative stabilities. Bench-top stability was checked for each analyte by spiking extracted blank urine samples at 2.0 μg/ml. The concentration of the acyl hydrazides never fell outside the range 90–110% deviation from zero time value over a period of 10 h. In addition, the stability of MTX and INDO derivatives were assessed by comparing the responses obtained from stored aqueous samples at 4 ± 1 °C to those obtained from freshly prepared derivatives after 1

and 3 days. For both derivatives the RSD never exceeded 3%.

3.5. Method applicability

QC samples at 3 concentration levels were prepared in replicates and analyzed. At least 66.66% of the QCs were within ±15% of their respective true concentrations, and less than 33.33%, excluding replicates at the same level, were outside these limits, well complying with the regulatory guidelines [26]. To express the relevance of the proposed method to real life situations, a human male volunteer was administered the studied compounds at levels derived from therapeutically relevant dosages (Section 2.7). The urinary excretion of MTX and INDO was monitored using the presently described methodology. At low to moderate doses, about 80% of a given dose of MTX is excreted unchanged in urine – the major part in the first 10 h after administration – following biphasic elimination kinetics with mean elimination half-lives of 2 and 8 h [27]. For INDO, 1/7 of an oral dose is excreted unchanged in urine within 24 h [28]. Results compiled in Table 2 show that after 10 h, about 73.4, and 12.3% of the administered doses of parenteral MTX and oral INDO were recovered in urine respectively. At the given dose levels, these values are reasonably consistent with previously reported data [28,29]. A real life chromatogram is depicted in Fig. 5.

In order to further prove the applicability of the described method we aimed at determining other NSAIDs as well. DIC similarly reacted with the hydrazine reagent in presence of EDC.HCl yielding a violet color derivative. This appeared at a t_R of 5.3 min using the prescribed chromatographic conditions.

Overall, the proposed method succeeded to achieve its goal in real samples. Sequentially, we report the first efforts to simultaneously determine MTX and INDO levels in human urine at different time intervals using a selective and validated SPE-HPLC methodology.

4. Conclusions

Combinations of antineoplastics and adjunct drugs are imperatively demanded to effectively suppress malignant cells or modify multifocal disease conditions. MTX is frequently and increasingly combined with NSAIDs to reduce pain or enhance the anticancer potential during chemotherapy or to control autoimmune inflammatory disorders. An urge to investigate the effect of NSAIDs on MTX excretion and vice versa prompted us to develop an analytical methodology to simultaneously monitor MTX and NSAIDs in urine. An adequately validated HPLC-DAD assay combining SPE with pre-column derivatization was designed to determine MTX and INDO in human urine. The described analytical tool provided some insights into the levels of urine MTX and INDO in a male volunteer. We hope that further studies concerning the urinary levels of MTX and NSAIDs in cancer, RA and psoriasis patients will shed more light on the therapeutic compatibility of such a promising combination remedy.

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