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Determination of dihydroergocryptine in human plasma and urine samples using on-line sample extraction–column-switching reversed-phase liquid chromatography–mass spectrometry

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Abstract

A rapid and sensitive assay for the determination of dihydroergocryptine (DHEC) in human plasma and urine samples with dihydroergotamine (DHET) as the internal standard was developed. The procedure employs on-line sample preparation using an extraction pre-column and an octadecylsilylsilica (ODS) analytical column. After centrifugation human plasma or urine were injected onto the pre-column, concentrated and extracted, back-flushed onto the analytical column and eluted with a binary methanol—aqueous formic acid gradient. Either determination of DHEC as well of its mono- and dihydroxy-metabolites was performed by measurement of the signal responses from MS detection in the selected reaction monitoring (SRM) mode using the transition of the respective parent ions to the common daughter ion at $m/z = 270.2$ amu. The limit of quantitation (LOQ) for determinations of DHEC in both plasma and urine were 25 pg/ml for injected sample volumes of 400 μl. Proportionality of signal responses versus concentration was accomplished within the range of 25–1000 pg/ml. Recovery of target analyte from plasma was 99%. Mean values of the coefficients of variation (CV) for the target analyte in plasma ranged from 1.7 to 13.8% (within-day) and 5.0 to 9.1% (between-day) and accuracy from 91.7 to 102.6% for the within-day and from 95.8 to 98.8% for the between-day measurements. The corresponding values for determinations in urine were 1.7–14.5% (within-day) and 5.3–11.8% (between-day) for CV and 95.8–110.7% (within-day) and 100.1–104.6% (between-day) for accuracy.

Keyword: *Dihydroergocryptine*

1. Introduction

The dopamine receptor agonist dihydroergocryptine (DHEC), which is metabolized to its DHEC monohydroxy- and DHEC dihydroxy-derivative (Fig. 1a–c), represents the hydrogenated derivative of ergocryptine and chemically belongs to the family of ergoline compounds. These are characterized by both a lysergic acid and a cyclic tripeptide moiety linked together by an amide bond. It shows very close structural similarity with bromocriptine, another dopamine receptor agonist differing from DHEC only by the hydrogenated Δ^{9-10} double bond and replacement of a hydrogen by a bromine atom in the indole moiety of the molecule [1]. DHEC mainly acts on the D_2-receptor but at least partially also exerts agonistic properties at the D_1-receptor [2]. For this reason it proves as a powerful remedy for treatment of Parkinson's disease either as monotherapeutic administration or in combination with levodopa [3,4].

As being the case for other ergot alkaloids, DHEC is subjected to extensive first-pass metabolism in the liver [5,6]. The major pathway of biotransformation occurs by hydroxylation at the pyrrolidine ring of the tripeptide moiety [7] as also observed for dihydroergotamine (DHET) [8].

Hitherto available data from structurally-related compounds imply involvement of the cytochrome P450 system in the oxidative pathway when passing the liver [1,9]. This

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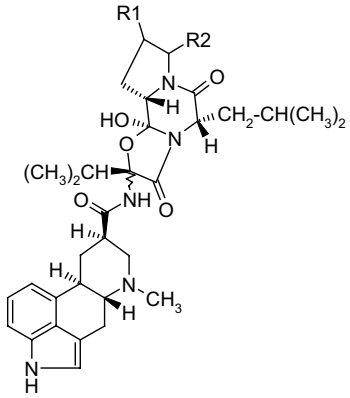

Figure 1a: R_1 = H R_2 = H
Figure 1b: R_1 = H R_2 = OH
Figure 1c: R_1 = OH R_2 = OH

Fig. 1. (a–c) Structural formula of DHEC (a), monohydroxy-DHEC (b) and dihydroxy-DHEC (c).

family of oxidizing enzymes composes of several isoforms with different substrate specificities [10] and is mainly responsible for the metabolism of a vast number of drugs [11,12]. It has been shown in the past that bromocriptine and other derivatives of the ergot alkaloid family undergo biotransformation by action of cytochrome oxidase P450 3A4 (CYP3A4) [1,13,14]. Recently a paper reporting on in-vitro identification of the cytochrome P450 isoforms responsible for the metabolism of dihydroergocryptine was published [15]. Although both DHEC and bromocriptine are inhibitors of CYP3A4 themselves [15,16] bioavailability of DHEC was nevertheless markedly increased by co-administration of erythromycin, a macrolide antibiotic, which acts as a strong CYP3A4 inhibitor [17].

Due to their low concentrations in biological material often being in the low ng/ml or even pg/ml range, extremely sensitive detection methods are needed. For this reason, either radioimmunological methods (radioimmunoassay, RIA) [9,17–22] and enzyme immunoassays (EIA) [17,23] have been preferentially used in the past. However, liquid chromatographic procedures, coupled to mass spectroscopic detection (MSD), provide some crucial advantages, in particular with concern to specificity. Indeed, high performance liquid chromatographic methods [1,6–9,13,15–17,24–30] prevail in analytical investigations of ergot alkaloids targeted either to get more precise insight into the metabolic pattern or to obtain thorough information of their concentrations in a biological environment, such as, e.g., serum or plasma, urine, tissue, etc. Among them three methods apply the selective "*on-line*" LC–MS hyphenation [13,15,17] allowing direct and unequivocal structural assignment of the compounds of interest even in those cases where co-elution of two or more species occurs provided ionization energy is not quantitatively consumed by the interfering ones.

The aim of the present study was to develop an analytical tool for accurate determination of DHEC concentrations in plasma and urine with dihydroergotamine as the internal standard (Fig. 2). For this purpose, an efficient procedure based on pre-column on-line sample extraction, subsequent chromatographic separation and final detection exploiting the great potential of the selective MSD principle (LC–MS coupling) in the selected reaction monitoring (SRM)-MS mode was chosen.

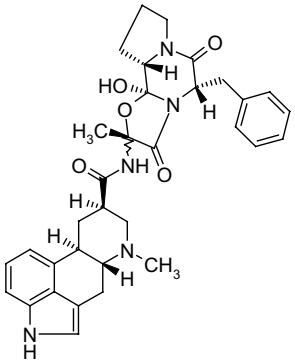

Fig. 2. Structural formula of internal standard dihydroergotamine (DHET).

2. Experimental

2.1. Reagents and materials

Dihydroergocryptine, used as its mesylate salt in all quantitive determinations and sold under the trade name Almirid^{R}, was a gift from Desitin Pharmaceutical Company (Hamburg, Germany). Dihydroergotamine applied as the internal standard was obtained from Tocris Cookson (Bristol, UK). Methanol (gradient grade for HPLC), 2-propanol (gradient grade for HPLC), ammonium acetate (p.a.), formic acid (p.a.) were from Merck (Darmstadt, Germany) and ammonium hydrogen carbonate was purchased from Fluka (Neu-Ulm, Germany). Ultrapure water for the use in HPLC experiments was prepared with a Milli-Q water system^{TM} from Millipore-Waters (Milford, MA, USA). A BioTrap 500 MS^{TM} pre-column (20 mm × 4 mm i.d.) from Chromtech (Hägersten, Sweden) and an Ultrasphere ODS (250 mm × 2 mm i.d., 5 μm particle size) analytical column from Beckman (Fullerton, CA, USA) were used for sample extraction and chromatographic separation, respectively.

2.2. Preparation of calibration samples, internal standard and samples

A stock solution of dihydroergotamine used as the internal standard was prepared at the 1000 ng/ml level in 10 mM formic acid–2-propanol 95:5 (v/v) and further diluted with the same solvent to yield a final concentration of 1000 pg/ml when 100 μl are added to volume aliquots of either 1000 μl blank plasma or urine. For preparation of the calibration curve dihydroergocryptine mesylate was dissolved in the

same solvent and diluted in such a way that final concentrations of 25–50–75–100–250–500 and 1000 pg/ml (related to DHEC free base) are obtained when 100 μl are added to volume aliquots of either 1000 μl plasma or urine. For assay validation three different concentrations used as samples yielding final concentrations of 100, 250 and 500 pg/ml when 100 μl are added to volume aliquots of either 1000 μl blank plasma or urine were prepared correspondingly.

Chilled plasma aliquots of 1000 μl drawn into heparinised tubes were allowed to thaw to room temperature. To each plasma sample prepared in this manner 100 μl of internal standard solution and 100 μl of both calibrator and sample solutions (see above) were added and thoroughly vortexed, filtered using 0.22 μm Millex GV_{13} filters of 13 mm diameter from Millipore (Eschborn, Germany) and centrifuged at 6000 × *g* for 1 h at 4 °C. For preparation of urine the same procedure with a volume of 1000 μl as described for plasma was used, except that samples were only centrifuged at 6000 × *g* for 15 min at 4 °C. In most cases DHEC levels in urine were far above the range of the calibration curve and therefore the samples were diluted with control urine obtained from healthy persons not subjected to DHEC treatment. Plasma and urine specimens prepared in this manner were stored at −20 °C prior to use.

2.3. Pre-column sample extraction and chromatographic separation

Aliquots (400 μl) of either plasma or urine, prepared as described in the previous subsection, were subjected to an identical pre-concentration procedure as that one previously reported for lonazolac [31] and 6β-testosterone [32] using the BioTrap 500 MS™ pre-column. Extraction was performed with 10 mM ammonium hydrogen carbonate–2-propanol 90/10 (v/v) at 3.2 ml/min. Back-flush of analyte onto the analytical C18 column and subsequent chromatographic separation was accomplished in the gradient mode with mobile phase A composed of 10 mM formic acid–methanol 90:10 (v/v) and mobile phase B composed of methanol–2-propanol 60:40 (v/v) at a flow-rate of 0.2 ml/min HPLC separation was started at $t = 0$ min with 90% mobile phase A and 10% mobile phase B and run isocratically for 4 min. After 2.5 min back-flush of sample at the same isocratic conditions was effected for 1.5 min via valve-switching by the mobile phase followed by gradient elution for 9 min to 10% mobile phase A and 90% mobile phase B and an isocratic hold at the latter conditions for another 7 min (see also Table 1). The column was washed with methanol–2-propanol 60:40 (v/v) and re-equilibrated

Table 1
Different time-dependent analytical events used in the column-switching procedure

Time (min)	Analytical events on PC-1/AC-1	Analytical events on PC-2/AC-2	MS/MS events
0–2.5	Sample injection-sample extraction on PC-1 with 10 mM ammonium hydrogen carbonate–2-propanol (90:10, v/v) by means of pump C at 3.2 ml/min	Start cleaning of AC-2 with methanol–isopropanol 60:40 (v/v) by means of pump B at 0.2 ml/min	Cleaning ion-source with methanol–water 1:1 at a flow-rate of 0.2 ml/min by means of pump E
2.5–4.0	Back-flush PC-1/AC-1 and begin of analytical separation with pump A at 0.2 ml/min (conditions see Section 2)	Cleaning loop and PC-2 by means of pump D with methanol–water 1:1 at 0.2 ml/min Cleaning of AC-2 with methanol–isopropanol 60:40 (V/V) by means of pump B continued	Cleaning ion-source continued
4.0–9.0	Analytical separation on AC-1 continued	Cleaning loop and PC-2 continued End of cleaning AC-2 (8.0 min) and start equilibration of AC-2 with 90% 10 mM formic acid–methanol (90:10) and 10% methanol–isopropanol (60:40) at 0.2 ml/min by means of pump B	
9.0–16.5	Analytical separation on AC-1 continued	Cleaning loop and PC-2 continued Equilibration of AC-2 continued	*Scan event* 1: SIM at *m/z* 584.2 (I.S.), 578.2 (DHEC), 594.2 (OH-DHEC), 610.2 (2OH-DHEC) *Scan event* 2: SRM on *m/z* 578.2 → 270.2 *Scan event* 3: SRM on *m/z* 594.2 → 270.2 *Scan event* 4: SRM on *m/z* 610.2 → 270.2
16.5–19.0	Analytical separation on AC-1 continued	Equilibration of PC-2 with 10 mM ammonium hydrogen carbonate–2-propanol (90:10, v/v) by means of pump C at 3.2 ml/min Equilibration of AC-2 continued	
19.0–20.0	End of analytical separation	End equilibration of PC-2 End equilibration of AC-2	Cleaning of ion-source by means of pump E

Pump A: elution pump; pump B: cleaning and equilibrating pump; pump C: extraction pump; pump D: cleaning pump; pump E: ion source cleaning pump.

with 90% mobile phase A and 10% mobile phase B while the other was running in the separation mode (for details see Table 1). Signal monitoring and quantitative determination was done by MS detection (conditions see below). The time events and experimental conditions of sample extraction on the pre-column, back-flush and chromatographic separation including the steps of individual re-equilibration of either pre-columns or analytical columns are depicted in Table 1, whereas the column-switching scheme is given in Refs. [31,32].

2.4. LC–MS conditions

The LC–MS/MS analysis was carried out on a type LCQ^{TM} ion-trap mass spectrometer purchased from ThermoQuest Finnigan (Bremen, Germany) equipped with an electrospray ionization (ESI) source operated in the selected reaction monitoring mode for genuine DHEC, its mono- and di-hyroxylated metabolite and in the selected ion monitoring (SIM) mode for DHET as the internal standard. Mass spectra were obtained in the positive ion mode applying a source voltage of 3.5 kV. The heated capillary temperature was set up to 260 °C, while the setting for the sheath gas (high purity nitrogen, 99.999%) was adjusted to 60 units. Data acquisition was done with the LC$_{Quan}$^{TM} part of the Xcalibur software from ThermoQuest-Finnigan by measuring the transition *m/z* 578.2 ⇒ 270.2 (relative collision energy 22%) for genuine DHEC (scan event 1), *m/z* 594.2 ⇒ 270.2 (relative collision energy 21.5%) for the monohydroxy-metabolite of DHEC (scan event 2) and *m/z* 610.2 ⇒ 270.2 (relative collision energy 21%) for the dihydroxy-metabolite of DHEC (scan event 3). For the internal standard DHET the ion of the protonated molecule at *m/z* 584.2 was used. The proportional conversion was calculated by using the measured peak area of DHEC and its mono- and dihydroxy-metabolites obtained in the SRM mode versus peak area of DHET as the internal standard obtained in the SIM mode. In order to protect the ion-trap source from the vast excess of contaminating compounds attributable to sample matrix components, the column efflux was diverted to waste for the first 10 min, whereas MS responses were acquired between 10 and 19.9 min, i.e., within the time interval where the compounds of interest were leaving the chromatographic support.

3. Results

3.1. Pre-column sample extraction, chromatographic separation and detection

The on-line sample preparation technique, as already been successfully applied for analyte enrichment of lonazolac [31] and 6β-hydroxytestosterone [32] from cell culture media, also proved to be advantageous in the present investigations. It permits rapid sample processing completely circumventing time consuming "*off-line*" alternatives often accompanied with marked sample loss and sometimes also partial sample degradation. Therefore, either final separation or detection is markedly facilitated, because the detrimental influence of possible interfering components of the matrix is strongly suppressed. Furthermore, owing to the use of a tandem pre-column/analytical column (PC/AC) system [31], pre-column 2 and analytical column 2 (PC/AC set 2) are equilibrated during pre-column extraction and analytical separation on PC/AC set 1, which additionally saves time and provides high sample throughput. For more detailed information about the principle of the used technique the interested reader is referred to Ref. [31].

As shown in Figs. 3a–d and 4a–d, depicting the selected reaction monitoring traces of DHEC (Figs. 3a and 4a), monohydroxy-DHEC (Figs. 3b and 4b), dihydroxy-DHEC (Figs. 3c and 4c) and the selected ion monitoring (SIM) trace of DHET (Figs. 3d and 4d) as the internal standard in plasma and urine, respectively, the signals of the target compound, its two hydroxy-metabolites and the internal standard show similar retention times and thus are not baseline resolved. Nevertheless, the HPLC assay provides satisfactory results due to sufficiently different transitions of either drug or metabolites. Therefore, the selective SRM/MS technique confers an optimum degree of reliability and accuracy to the method of quantitative determination and thus reliable results are obtained.

3.2. Specificity

MS detection in the SRM mode provides an optimum degree of specificity in either plasma or urine by measurement of the transitions *m/z* 578.2 ⇒ 270.2 for DHEC, *m/z* 594.2 ⇒ 270.2 for monohydroxy-DHEC and *m/z* 610.2 ⇒ 270.2 for dihydroxy-DHEC. Therefore, quantitation of DHEC can be accomplished without any impairment from interfering compounds.

3.3. Recovery

Recovery of DHEC in plasma by use of the BioTrap 500 MS^{TM} pre-column was 99%. Due to the fact that urine is the much less complex biological matrix compared with plasma, recovery of DHEC has not been determined in the present study but in advance, similar or even better values than those achievable with plasma are to be expected. This point of view is also corroborated by the convincing data for either precision or accuracy (see Table 3), which are in a comparable range with those from DHEC determinations in plasma.

3.4. Proportionality of signal responses versus concentration

For determination of DHEC in either plasma or urine in the range of 25–1000 pg/ml including seven standards

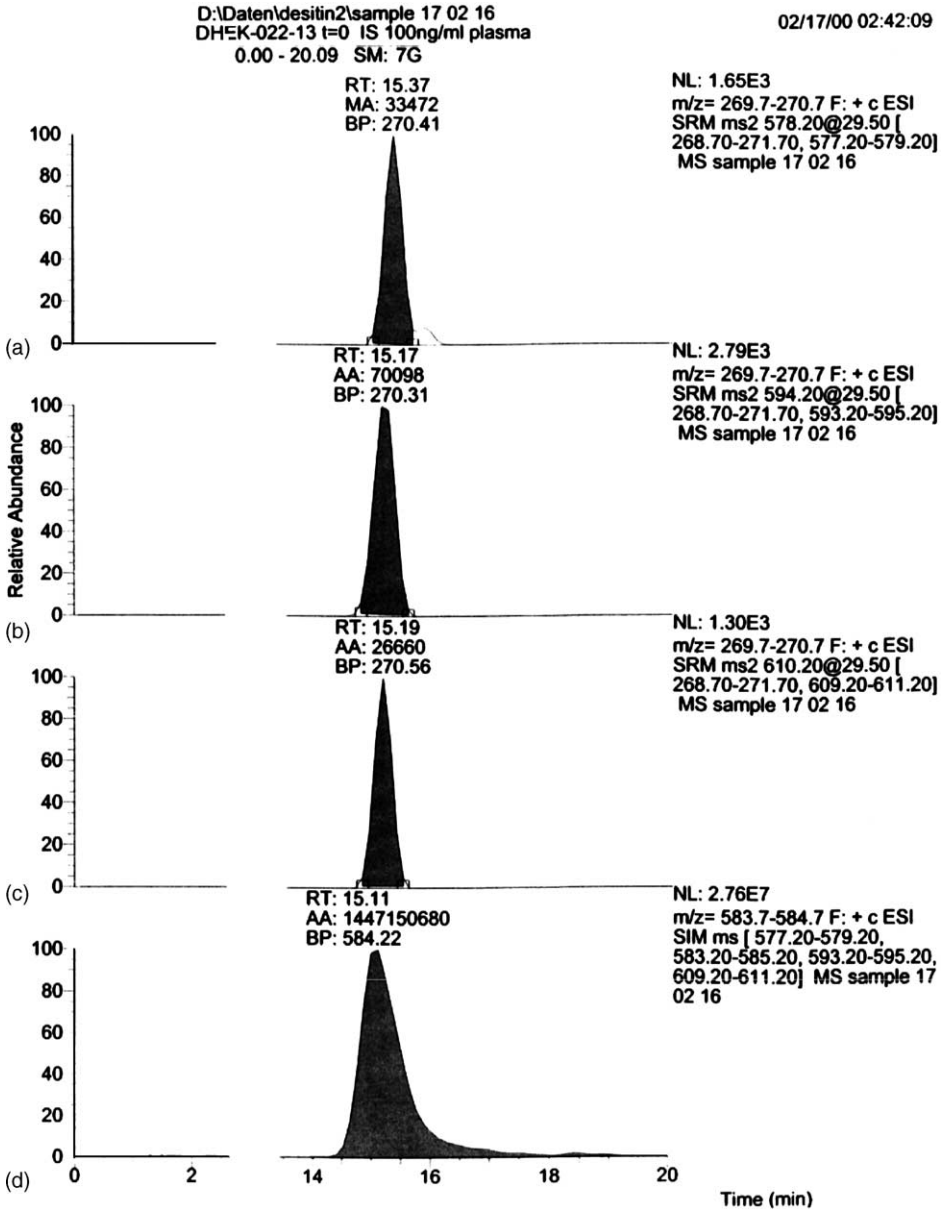

Fig. 3. (a–d) LC–MS(SRM) traces of DHEC (a), monohydroxy-DHEC (b), dihydroxy-DHEC (c) and the LC–MS(SIM) trace of DHET (d) in a human plasma sample.

(i.e., 25–50–75–100–250–500 and 1000 pg/ml) and DHET as the internal standard, a quadratic fit of the peak areas from the respective SRM(SIM)/MS responses versus concentration proved to be feasible for reliable DHEC determination. In all cases the mean values for the correlation factor R^2 were >0.999 ($n = 6$). Although not available as pure compounds, the mono- and dihydroxy-metabolites were included in quantitative determinations. Concentrations were calculated with the DHEC calibration curve on the basis of the transitions m/z 594.2 $\Rightarrow$ 270.2 for monohydroxy-DHEC and m/z 610.2 $\Rightarrow$ 270.2 for dihydroxy-DHEC both yielding the same fragment ion as the parent compound.

3.5. Limit of quantitation

The limit of quantitation (LOQ) of DHEC in both human plasma and urine was 25 pg/ml at sample volumes of 400 μl.

3.6. Precision and accuracy

Precision and accuracy data of DHEC in plasma have been determined in quintuplicate at the 100, 250 and 500 pg/ml level at three different days. As can be seen from Table 2, the values for the within-day coefficients of variation (CV) and accuracy, respectively, measured at three different days ($n = 5$) ranged from 2.8 to 13.9% and 97.2 to 99.8% for

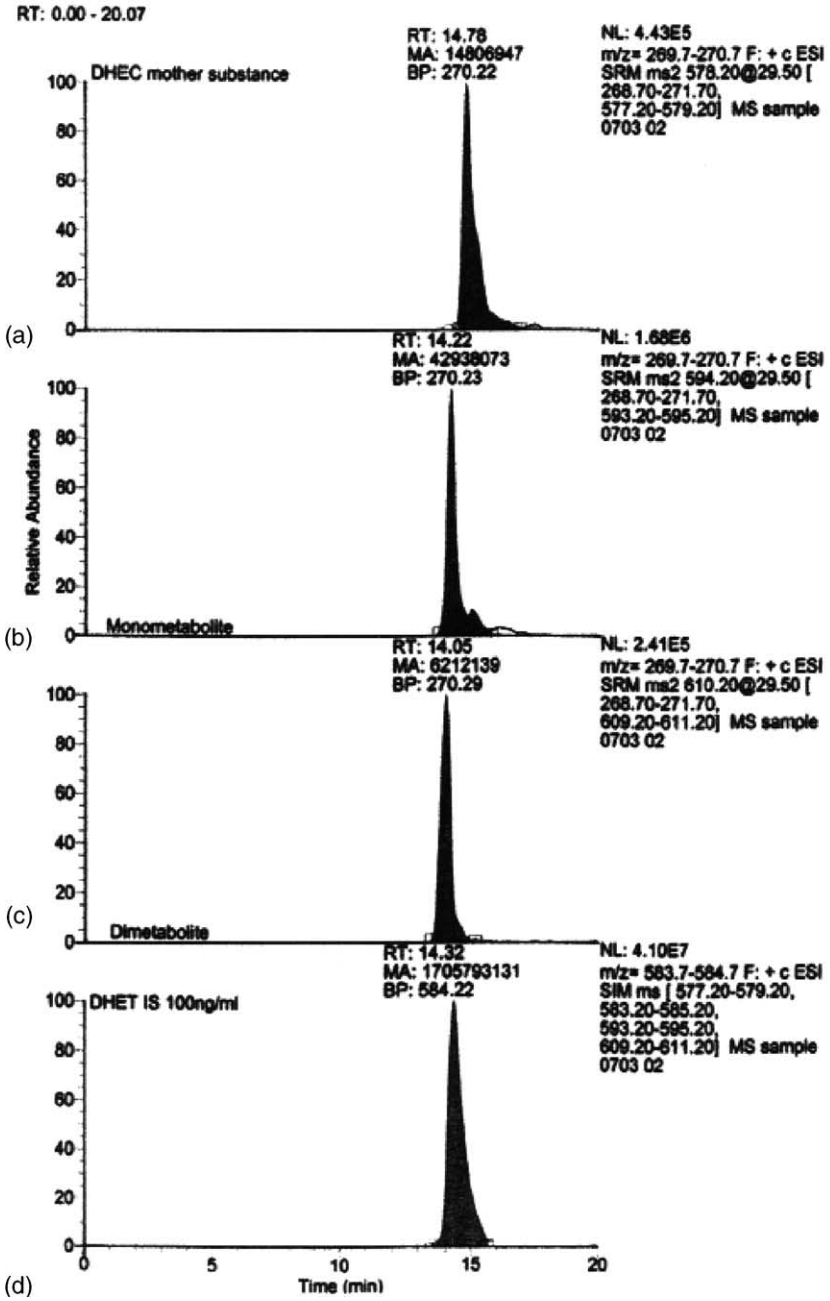

Fig. 4. (a–d) LC–MS(SRM) traces of DHEC (a), monohydroxy-DHEC (b), dihydroxy-DHEC (c) and the LC–MS(SIM) trace of DHET (d) in a human urine sample.

100 pg/ml, from 1.7 to 8.0% and 94.2 to 99.0% for 250 pg/ml and from 1.7 to 8.5% and 91.7 to 102.6% for 500 pg/ml, respectively. The corresponding between-day CV and accuracy values measured at three different days, as also shown in Table 2, were 9.1 and 98.6% for 100 pg/ml ($n = 15$), 5.0 and 95.8% for 250 pg/ml ($n = 15$) and 5.6 and 97.4% for 500 pg/ml ($n = 14$).

Table 3 shows the values for the within-day coefficients of variation (CV) and accuracy, respectively, measured in urine at three different days ($n = 7$) and ranging from 7.8 to 14.5% and 97.9 to 110.7% for 100 pg/ml, from 2.8 to 7.2% and 101.3 to 104.9% for 250 pg/ml and from 1.7 to 8.1% and 95.8 to 104.6% for 500 pg/ml, respectively. The corresponding between-day CV and accuracy values measured at three different days, as also shown in Table 3, were 11.8 and 104.6% for 100 pg/ml ($n = 21$), 5.3 and 102.5% for 250 pg/ml ($n = 21$) and 6.3 and 100.1% for 500 pg/ml ($n = 21$).

3.7. Freeze and thaw stability

When both plasma and urine samples were subjected to different freeze-thaw cycles, i.e., thawing from −20 °C to room temperature, no detrimental effect was observed and

Table 2
Determination of within-day and between-day data for precision and accuracy for plasma samples

Day 1	Day 2	Day 3	Between-day data
Within-day data			
100 pg/ml			
Mean ($n = 5$): 98.8; S.D.: ±2.77%; CV: 2.80%; accuracy: 98.8	Mean ($n = 5$): 99.8; S.D.: ±13.83%; CV: 13.86%; accuracy: 99.8	Mean ($n = 5$): 97.2; S.D.: ±8.84%; CV: 9.1; accuracy: 97.2	Mean ($n = 15$): 98.6; S.D.: ±9.0%; CV = 9.1%; accuracy: 98.6
250 pg/ml			
Mean ($n = 5$): 235.6; S.D.: ±18.74%; CV: 7.96%; accuracy: 94.2	Mean ($n = 5$): 247.4; S.D.: ±4.28%; CV: 1.73%; accuracy: 99.0	Mean ($n = 5$): 235.8; S.D.: ±4.21%; CV: 1.78%; accuracy: 94.3	Mean ($n = 15$): 239.6; S.D.: ±12.0%; CV = 5.0%; accuracy: 95.8
500 pg/ml			
Mean ($n = 5$): 512.8; S.D.: ±8.9%; CV: 1.74%; accuracy: 102.6	Mean ($n = 5$): 484.4; S.D.: ±19.41%; CV: 4.0%; accuracy: 96.9	Mean ($n = 4$): 458.5; S.D.: ±21.2%; CV: 4.6%; accuracy: 91.7	Mean ($n = 14$): 487.1; S.D.: ±27.4%; CV = 5.6%; accuracy: 97.4

Table 3
Determination of within-day and between-day data for precision and accuracy for urine samples

Day 1	Day 2	Day 3	Between-day data
Within-day data (100 pg/ml)			
Mean ($n = 7$): 97.9; S.D.: ±7.82%; CV: 8.0%; accuracy: 97.9	Mean ($n = 7$): 110.7; S.D.: ±12.87%; CV: 11.62%; accuracy: 110.7	Mean ($n = 7$): 105.1; S.D.: ±14.5%; CV: 13.79; accuracy: 105.1	Mean ($n = 21$): 104.6; S.D.: ±12.4%; CV: 11.8%; accuracy: 104.6
Accuracy: 102.5 (250 pg/ml)			
Mean ($n = 7$): 253.3; S.D.: ±7.06%; CV: 2.79%; accuracy: 101.3	Mean ($n = 7$): 262.1; S.D.: ±18.76%; CV: 7.16%; accuracy: 104.9	Mean ($n = 7$): 253.1; S.D.: ±13.37%; CV: 5.28%; accuracy: 101.3	Mean ($n = 21$): 256.2; S.D.: ±13.5%; CV: 5.3%; accuracy: 102.5
Within-day data (500 pg/ml)			
Mean ($n = 7$): 499.4; S.D.: ±8.56%; CV: 1.71%; accuracy: 99.9	Mean ($n = 7$): 523.1; S.D.: ±27.55%; CV: 5.27%; accuracy: 104.6	Mean ($n = 7$): 478.9; S.D.: ±38.63%; CV: 8.07%; accuracy: 95.8	Mean ($n = 21$): 500.5; S.D.: ±31.5%; CV: 6.3%; accuracy: 100.1

thus underlines the stability of the drug under real-life conditions.

4. Discussion

4.1. Sample preparation, chromatographic separation and detection

Despite being extremely sensitive, in particular when coupled to mass selective detection (MSD), gas chromatography [26] does not prove to be the method of choice due to both the analyte's low volatility and thermal sensitivity. Therefore, prior derivatization, e.g., by means of bis(trimethylsilyl) acetamide (BSA) in the reaction port of the gas chromatograph or *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) is required, as described for determination of lysergic acid diethylamide (LSD) [27]. In addition, also planar chromatographic techniques [26] do not meet the basic requirements of both separation efficiency and accuracy.

Although in terms of sensitivity, enzymatic and radioimmunological techniques, such as, e.g., EIA [17,23] and RIA [9,17–22] provide excellent sensitivity with limits of quantitation spanning the range from the lower ng/ml to lower pg/ml level, they very often are lacking specificity. As a consequence, more or less marked cross-reactions have to be taken into account but nevertheless "*group*" determinations may be accomplished. In contrast, only liquid chromatography proves to be the suitable alternative to solve the problems of separation efficiency and detection sensitivity. Indeed RP-HPLC, especially when using sensitive detection, is excellently suited for this purpose. Besides availability of a powerful separation system, an efficient sample preparation procedure is a further prerequisite for either concentration of analyte or minimization of interferences arising from matrix constituents, which otherwise may seriously affect both final chromatographic separation and detection. In order to fulfill these fundamental requirements, a variety of sample preparation, chromatographic separation and detection procedures is reported in the literature.

In this respect precipitation with acetonitrile is used for extraction of DHEC from rat and human hepatocyte cultures, from rat, monkey and human microsomes [6] as well as in cytochrome P450 enzyme inhibitor screening systems [16]. Acetone–methanol 3/1 (v/v) was chosen for extraction of ergot derivatives from V79 cells [14]. Liquid-liquid extraction with dichloromethane [1,8] and diethylether [13] was applied in the case of rat [1] and human [8,13] liver microsomal preparations. Solid-phase extraction (SPE) of dihydroergotamine in rabbit and human serum is described in Ref. [30]. Previous investigations in our laboratory [15] took advantage from SPE "*on-line*"—coupled to HPLC, a procedure described for the first time in 1981 [33], using so-called biocompatible cartridge systems, such as BioTrap^{TM} pre-column materials. For this reason, there was no question to exploit the potential of this powerful and time-saving technique during the course of the present investigations.

The fact that ergot alkaloids possess different amine functions requires the use of buffered mobile phase systems and performed by either isocratic elution [6,8,14,24–26,28–30] or in the gradient mode [1,7–9,13,15,16,25] on C_{18} and C_8 columns. In some applications acetonitrile–10 mM ammonium carbonate mixtures [1,9,13,24,25,28] were used but except Ref. [24] no indications are made with respect to long-term stability of the chromatographic support under the slightly alkaline conditions of the mobile phase. Other applications take advantage from aqueous solutions of acetonitrile containing ammonium carbaminate [7,8], ammonium acetate [6], formic acid–ammonium acetate buffer [15], triethylamine [24,29], 2-propanol–Na_2HPO_4 buffer [30], aqueous solutions of methanol containing NaH_2PO_4 [14], ammonium acetate [24], triethylamine [24], and acetic acid [16]. Bare silica gel as the chromatographic support and a dichloromethane–methanol 95.5 (v/v) mobile phase was described in Ref. [8]. In investigations employing both bare silica gel and reversed-phase materials, such as, e.g., C_{18}, C_8 and C_2 supports, chloroform–methanol, chloroform–ethanol and chloroform–*n*-hexane–methanol mobile phases were used on bare silica gel, whereas acetonitrile–ammonium carbonate eluents are preferably applied on reversed-phase sorbents [26]. An extensive study addressing the influence of pH within the range of 2–13 on separation selectivity of dihydroergotoxine metabolites is given in Ref. [24].

For detection of ergot alkaloids measurement of the UV signal responses was applied at 254 and 305 nm [1], 280 nm [8,9,24,25], 300 nm [27], 320 nm [25,26] and despite a reported LOD of 25 ng/ml [25], sensitivity is still expected to be too low for most applications. Therefore, more sensitive alternatives, such as measurement of fluorescence responses [6,14,29,30] in general providing a gain in sensitivity of at least one order of magnitude over UV-detection are required. In this respect LOQ values of 0.2 ng/ml and 1 ng/ml were reported for determination of bromocriptine in plasma and vitreous aqueous humour, respectively, of the rabbit [29]. In rabbit and human serum LOD and LOQ values of 0.2 and 0.7 ng/ml, respectively, were found for determination of dihydroergotamine. Nevertheless, mass spectroscopy in the positive ion mode will be the method of choice for both unequivocal structural assignment and optimum detection sensitivity, because ergot alkaloids bear structural elements, strongly contributing to stabilization of positive charges. Although in Refs. [1,8,9] "*off-line*" mass spectroscopic investigations on ergot alkaloids are reported, the time-consuming technique of prior sampling and evaporation of mobile phase precludes the use in the case of high sample throughput in routine applications and therefore making on-line LC–MS coupling experiments [13,15,17] indispensable. However, in the latter cases no data were given with respect to the LOQ and LOD values.

During the course of method development in order to obtain a reliable and highly sensitive chromatographic procedure, we used a similar volatile mobile phase system as that reported in Ref. [16], except that formic acid was used instead of acetic acid. Mass selective detection of DHEC in the positive ion mode provides LOQ values in both human plasma and urine in the lower ng/ml to pg/ml range, as usually only observable with enzymatic and radioimmunological procedures. In addition to the structural assignment of DHEC by means of the SRM-MS technique (m/z 578 ⇒ 270.2) in either plasma or urine, the corresponding monohydroxy-metabolites (m/z 594 ⇒ 270.2) and dihydroxy-metabolites (m/z 610 ⇒ 270.2) metabolites could also be recognized unequivocally. Unfortunately, the non-availability of authentic samples of the two hydroxy-metabolites precluded their exact quantitation. Nevertheless, approximative determinations of them could be done by assuming that the SRM-MS transition energy of m/z 594 ⇒ 270.2 for Fig. 1b and m/z 610 ⇒ 270.2 for Fig. 1c are of comparable size as that of the parent compound Fig. 1a at m/z 578 ⇒ 270.2. In a first approximation this seems to be a reasonable assumption provided that the energy needed to cleave the amide bond linking together the lysergic acid and the tripeptide moiety is not expected to be essentially influenced by one or two hydroxy groups at the pyrrolidine ring far away from the cleavage site. As a consequence, it may be possible to quantify metabolites Fig. 1b and c on the basis of the DHEC standard curve. However, in order to either support or refute this assumption, the pure metabolites are required and therefore, results obtained in this way are to be considered with care.

4.2. Recovery, proportionality of signal response, sensitivity, precision and accuracy

As already previously reported in Section 3 and furthermore underlined by Tables 2 and 3, sufficient evidence is provided in favor of reliable and reproducible determination of DHEC in plasma and urine. The applied "*on-line*" column-switching RP-HPLC assay with MS detection yielded values for recovery from the biological matrix almost approaching the theoretical limit. Furthermore, sensitivity approximates concentrations, which otherwise are only measurable by means of extremely sensitive methods like EIA and RIA and also offers the advantage of the highest possible degree of specificity. Last but not least satisfactory data for precision and accuracy provide additional support for the presence of an efficient analytical technique that can be used for reliable determination of large sample numbers.

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