ELSEVIER

Journal of Chromatography B, 767 (2002) 245–253

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Quantitative liquid chromatography-mass spectrometry determination of isatin in urine using automated on-line extraction

Matthias Unger[a], Wolfgang Jacobsen[a], Ulrike Holzgrabe[b], Leslie Z. Benet[a,*]

[a]*Department of Biopharmaceutical Sciences, University of California, 533 Parnassus Ave., Room U-68, San Francisco, CA 94143-0446, USA*

[b]*Institut für Pharmazie und Lebensmittelchemie, Julius-Maximilians-Universitaet Wuerzburg, 97074 Wuerzburg, Germany*

Received 19 June 2001; received in revised form 30 October 2001; accepted 31 October 2001

Abstract

Here we describe a simple, fast and sensitive liquid chromatography/mass spectrometry method with automated on-line extraction to quantify isatin, an endogenous monoamine oxidase, and atrial natriuretic peptide inhibitor, in urine. After derivatisation of isatin to isatinoxime with hydroxylamine hydrochloride and zinc sulfate precipitation, samples were loaded on the extraction column, washed and, after activation of the column-switching valve, backflushed onto the analytical column. Using electrospray ionisation, $[M+H]^+$ ions could be detected in the selected ion monitoring mode. The assay was linear from 5 to 5000 ng/ml ($r^2>0.99$) and analytical recovery was >80%. Inter-assay precision for the quality control samples was less than 3% and inter-assay accuracy was within ±5%.

Keywords: Isatin; Isatinoxime; 5-Methylisatin; Indoxyl; Indican

1. Introduction

Today, high performance liquid chromatography (HPLC) is state of the art for the detection, identification and quantification of drugs [1,2]. In particular, the coupling with MS allows the sensitive and selective quantification of low sample amounts in crude matrices like urine, blood or tissues [3–5]. MS detection provides additional information for the identification and structure elucidation of known and unknown compounds and allows the selective detection of substances when selected ion monitoring (SIM) is applied [6,7].

Unfortunately, the high amounts of salts and/or proteins present in biological fluids and tissues require the use of additional purification steps including liquid/liquid extraction or solid-phase extraction [8,9]. A convenient method to avoid purification of samples prior to injection onto the HPLC column is the application of automated on-line extraction [10–12]. This technique, also called column switching, allows the easy and convenient purification of the sample by adsorption of the target compound on a small precolumn and finally elution onto the analytical column with running buffer in the backflush mode.

Isatin, an endogenous inhibitor of monamine oxidase (MAO) and atrial natriuretic peptide (ANP)

*Corresponding author. Tel.: +1-415-476-3853; fax: +1-415-476-8887.

E-mail address: benet@itsa.ucsf.edu (L.Z. Benet).

1570-0232/02/$ – see front matter
PII: S0378-4347(01)00538-2

was first detected and identified in urine as a component of tribuline, an endogenous MAO inhibitory substance [13,14]. Isatin is present in urine and blood of rats and humans but it can also be detected in the brain, kidney and heart [15]. Although its physiological function still remains unclear the relatively high amounts of isatin in rat brain [16] and human urine and plasma [17] point to a possible role of isatin in humans. Since isatin excretion in rat urine is increased in stress situations like food deprivation and acute cold exposure [18] there might be a correlation between urine isatin levels and the endogenous formation of isatin in tissues like brain, heart or kidney.

Isatin (2, 3-dioxoindole, Fig. 1) is a low molecular mass compound with a α, β-dicarbonyl function at C (2) and C (3) of the indole nucleus that is responsible for its bright orange colour. Various analytical methods have been applied to identify and quantitate isatin in biological fluids and tissues including ELISA [19], gas chromatography/mass spectrometry (GC–MS) [20], and HPLC with UV detection [17,21]. After extraction of tissues with hydrochloric acid (HCl) and acidification of urine or plasma samples with HCl, liquid/liquid extraction with ethylacetate was applied for further purification of samples [17,20,21]. The detection and quantification of isatin in plasma, urine and tissues of rats by GC–MS was performed after derivatisation of isatin to the corresponding oxime with hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$), solid-phase extraction with silica gel and finally derivatisation with MTBSTFA [20]. Manabe et al. [17] used HPLC with UV detection to quantify isatin in human plasma and urine. Because of the low selectivity of UV detection, solid-phase extraction and preparative HPLC purification were used to eliminate interfering matrix compounds. HPLC/UV was also applied to determine isatin in rat urine and kidney extracts by Hamaue et al. [21]. After liquid/liquid extraction with ethylacetate and evaporation of extracts with nitrogen the samples were redissolved in phosphate buffer and directly injected onto the HPLC system without further purification. Major drawbacks of this method are the lack of a suitable internal standard and a relatively low sensitivity of about 300 ng per ml urine. The latter is a clinically important deficiency since isatin levels in human urine samples have been reported to be as low as 18 ng/ml [17]. Another disadvantage that is shared by all methods involving a liquid/liquid extraction step is the addition of hydrochloric acid (HCl) to provide a quantitative extraction of isatin into the widely used ethylacetate. This acidification, which involves the addition of 4 *M* HCl [21] or even 6 *M* HCl [17], could hydrolyse unstable phase two metabolites of isatin or indoxyl that are thereby converted to isatin, thus suggesting a higher isatin level.

In order to quantify isatin in urine samples of healthy humans we used LC–MS with automated on-line extraction (LC/LC–MS), which allowed the simple, fast, sensitive and selective determination of isatin after derivatisation to the corresponding oxime using $NH_2OH{\cdot}HCl$. The derivatisation step and automated on-line extraction yielded a good recovery and sensitivity so that the tedious liquid/liquid extraction and preliminary pH adjustment with HCl could be avoided. Using MS detection in the SIM mode instead of conventional UV detection provided excellent sensitivity but also superior selectivity from interfering endogenous matrix compounds. Thus the method described here should also be suitable to measure isatin in urine samples of patients who are on various medications. To our knowledge, this is the first application of LC/LC–MS for isatin, 5-methylisatin and their oxime derivatives.

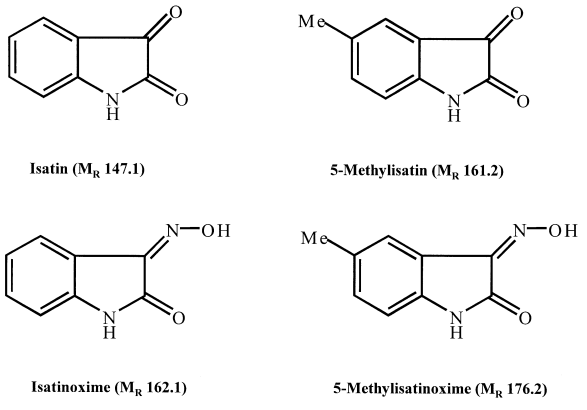

Fig. 1. Structures and molecular weights (M_R) of isatin, isatinoxime, 5-methylisatin and the internal standard 5-methylisatinoxime.

2. Experimental

2.1. Equipment

Samples were analysed on a Hewlett-Packard (HP, Palo Alto, CA, USA) LC/LC-mass selective detector system consisting of the following series 1100 HPLC components: HPLC I: G1311A quarternary pump, G1322A degasser and G1329A autosampler equipped with a G1330A thermostat; HPLC II: G1312A binary pump, G1322A degasser, G1316A column thermostat and G1946A mass selective detector. The two HPLC systems were connected via a 7240 Rheodyne six-port switching valve mounted on a step motor (Rheodyne, Cotati, CA, USA). The system was controlled and data were processed using the Hewlett-Packard (HP) ChemStation Software Revision A.07.01.

2.2. Chemicals

Zinc sulfate, 5-methylisatin, formic acid and $NH_2OH{\cdot}HCl$ were purchased from Sigma (St Louis, MO, USA). Isatinoxime was obtained from Lancaster (Windham, NH, USA) and isatin was purchased from Aldrich (Milwaukee, WI, USA). Methanol and water were of HPLC grade and obtained from Fisher Scientific (Fair Lawn, NJ, USA).

2.2.1. Synthesis of 5-methylisatinoxime

One hundred milligrams of 5-methylisatin and 430 mg of $NH_2OH{\cdot}HCl$ were dissolved in 200 ml of water and heated at 70°C for 3 h in a water bath. After cooling to room temperature the solution was stored at 4°C overnight. The resulting precipitate was sucked through a Buchner funnel and washed twice with small amounts (10 ml) of ice cold water and ice cold diethyl ether. Finally, the precipitate was dried at 50°C overnight and the resulting yellow powder (79 mg) was used without further purification. The substance revealed a purity of 97% determined by HPLC with Diode Array Detection (DAD) and the structure was confirmed using HPLC/DAD (UV maxima at 254 and 298 nm) and LC–MS (m/z of $[M+H]^+ = 177$).

2.3. Stock solutions

Stock solutions were prepared from isatinoxime (1000 μg/ml in MeOH) and 5-methylisatin (1000 μg/ml in MeOH) and diluted to the appropriate concentrations (100, 10 and 1 μg/ml) with water. The internal standard 5-methylisatinoxime was prepared by dilution of the stock solution (300 μg/ml in MeOH) to the corresponding concentration of 3000 ng/ml with water. For the final preparation of calibration standards urine was used instead of water.

2.4. Urine samples

Urine for development and validation of the assay was obtained from healthy volunteers and the creatinine content was determined using the method described by Jaffé [22]. All samples were collected in the morning and stored at −80°C.

2.5. Sample preparation

2.5.1. Calibration standards

For calibration, urine of five individuals was pooled and spiked with 5, 25, 100, 500, 1000, 2500 and 5000 ng/ml isatinoxime. Eight hundred microliters were mixed with 100 μl internal standard (3000 ng/ml 5-methylisatinoxime), 50 μl of water and finally 50 μl 0.2 *M* $ZnSO_4$ in water. Samples were vortexed and centrifuged at 11 000 *g* for 15 min.

2.5.2. Urine samples

Before analysis, the urine samples were thawed at room temperature and centrifuged at 11 000 *g* for 5 min. Urine (800 μl) was transferred into an Eppendorf tube and after the addition of 100 μl of an aqueous 5-methylisatinoxime solution (3000 ng/ml) and 50 μl $NH_2OH{\cdot}HCl$ (20 mg/ml in water) the sample was heated at 70°C for 1 h on a Dri-Bath (Barnstead/Thermolyne, Dubuque, IA, USA). After cooling to room temperature and addition of 50 μl of an aqueous solution of zinc sulfate (0.2 *M*), samples were vortexed and centrifuged at 11 000 *g* for 15 min. Two-hundred microliters of the supernatant were transferred into HPLC screw cap vials with 300-μl inserts (HP).

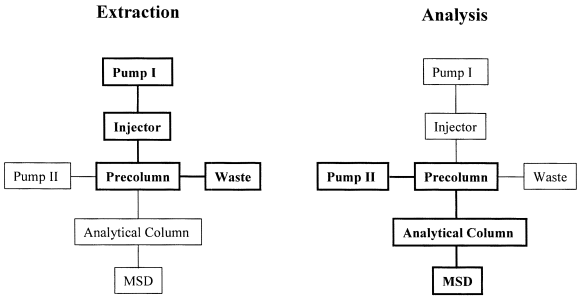

Fig. 2. Schematic drawing of the instrumental set-up using column switching. Extraction: pump I, injector, precolumn, waste; Analysis: pump II, precolumn, analytical column, mass selective detector (MSD).

2.5.3. Linearity of the derivatisation procedure

Different concentrations of 5-methylisatin in urine (5, 25, 100, 500, 1000, 5000 and 10.000 ng/ml) were derivatised with $NH_2OH \cdot HCl$ and prepared as described above. An aqueous isatinoxime solution (2500 ng/ml) was used as the internal standard instead of 5-methylisatinoxime.

2.6. LC/LC–MS analysis

One hundred microliters of the samples were injected onto a HP 20×4 mm extraction column filled with Hypersil ODS, 5 μm particle size (Shandon, Chadwick, UK). Samples were washed with a mobile phase of 0.02% formic acid and the flow-rate of the loading buffer was 3 ml/min. The temperature for the extraction column and the analytical column was set to 25°C. After 2 min, the switching valve was activated and the analytes were eluted from the extraction column onto the 150×4.6 mm C_8, 3.5 μm analytical column (Zorbax XDB C_8, HP) in the backflush mode (Fig. 2). The mobile phase consisted of methanol (A) and 0.02% formic acid (B) and the flow-rate was 0.5 ml/min. The following gradient was run: time 0 min: 45% (A), 5 min: 45% (A), 10 min: 50% (A), 15 min: 50% (A), 20 min: 60% (A), 22 min: 60% (A), 23 min 100% (A), 28 min 100% (A), 29 min: 45% (A), 34 min: 45% (A). Two minutes after sample injection, the mass selective detector was activated. Settings of the mass selective detector are listed in Table 1.

2.7. Method validation

2.7.1. Calibration curve

For calibration of isatinoxime in urine, six samples of each concentration (refer to Section 2.5.1) were measured. The correlation coefficients for isatinoxime calibration and the derivatisation procedure of 5-methylisatin were assessed using the regression analysis implemented in the Microcal Origin software (version 3.5, Microcal Software Inc., Northampton, MA, USA).

2.7.2. Recovery

Recoveries were calculated from average peak area ratios obtained after analysis of three quality

Table 1
Mass selective detector settings

Parameter	
Capillary exit voltage (fragmentor)	+60 V
Capillary voltage (V_{cap})	−4000 V
Ion energy (octopole)	+5 V
Nebulizer gas	Nitrogen, purity 5.0 Pressure: 50 p.s.i.
Drying gas	Nitrogen, purity 5.0 Temperature: 300°C Flow: 10 l/min
Quadrupole temperature	100°C
Dwell time	589 ms
Scan range (full scan mode)	100–300 amu
Extracted ions (SIM mode)	m/z=163 (isatinoxime) m/z=177 (5-methylisatinoxime)

1 p.s.i.=6894.76 Pa.

control samples of isatinoxime (50, 750 and 4000 ng/ml) in urine and in water ($n=3$). The mass spectrometer responses of the extracted samples (isatinoxime in urine, using $ZnSO_4$ precipitation and column switching) were compared with the responses after injection of the corresponding standard solutions (isatinoxime in water) directly onto the analytical column, bypassing the extraction column.

2.7.3. Inter-assay precision and accuracy

Inter-assay (day-to-day) precision and accuracy were evaluated by analysis of the aforedescribed (Section 2.7.2) quality control samples on three different days.

3. Results and discussion

3.1. Method development

When analysed with C_8 or C_{18} reversed-phase stationary phases using water–methanol gradients isatin and 5-methylisatin (for structures and molecular masses of compounds, see Fig. 1) showed severe fronting (Fig. 3) possibly due to complexation of the α, β-dicarbonyl function with metal ions or additional strong polar interactions of the carbonyl group at C (3) with silanol groups of the stationary phase. This explanation is supported by the fact that symmetrical peak shapes were obtained for the corresponding oximes as can be seen in Fig. 3. Although isatin is a very weak base and cannot be protonated or deprotonated under the conditions normally applied for reversed-phase chromatography we decided to use ESI in the positive ion mode for MS detection. After addition of various amounts of formic acid (0.01/0.02/0.05 and 0.1%) to the running buffer we obtained a detection limit of about 50 ng/ml for isatin using water with 0.02% formic acid and MeOH (data not shown).

Fig. 3 shows the LC–MS analysis of isatin, 5-methylisatin, isatinoxime and 5-methylisatinoxime (each concentration 10 μM) using full scan mode. The presence of an additional methyl group in position 5 of the phenyl ring resulted in a marked increase in retention time for 5-methylisatin and 5-methylisatinoxime compared to isatin and isatinoxime, respectively.

The intensities of the $[M+H]^+$ signals were somewhat higher than the signal intensities of the corresponding sodium adducts $[M+Na]^+$ when a fragmentation voltage of 60 V was used (Fig. 4). The application of higher fragmentation voltages (75/90 V) resulted in improved intensities for the proton adducts compared to the $[M+Na]^+$ signals but led to a decreased sensitivity. Also lower fragmentation voltages (30/45 V) did not increase the sensitivity because the intensity of the the $[M+H]^+$ signal was much lower (data not shown). Interestingly, the intensity of the sodium adducts for isatinoxime and 5-methylisatinoxime (ca. 85% relative abundance, Fig. 4) were higher than the signal intensities of the sodium adducts of their parent compounds (ca. 70% relative abundance, Fig. 4).

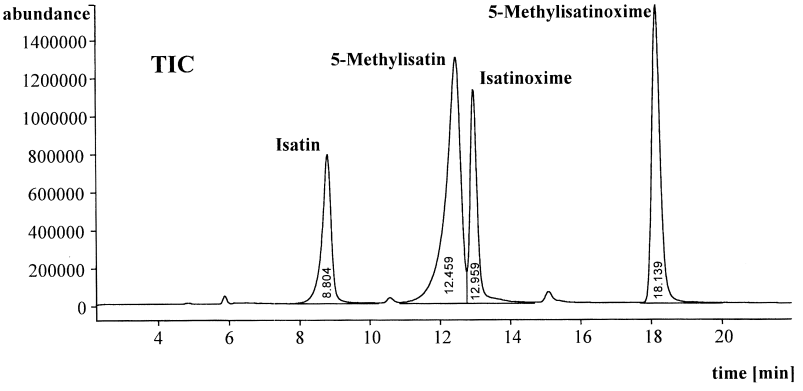

Fig. 3. Total Ion Current (TIC) of the LC/LC–MS analysis of isatin, 5-methylisatin, isatinoxime and 5-methylisatinoxime (concentration 10 μM each) in full scan mode; (for experimental details, see Section 2.6).

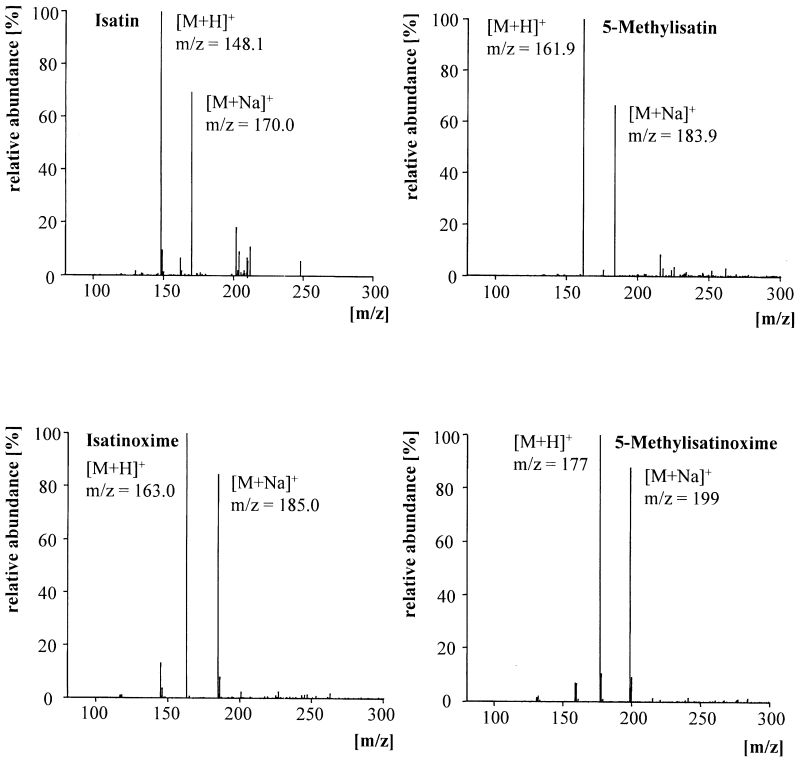

Fig. 4. Full scan mass spectra of compounds analysed in Fig. 3. Capillary exit voltage (fragmentation voltage) 60 V (for mass selective detector settings refer to Table 1).

Due to the strong peak asymmetry and the presence of interfering matrix components when analysed in urine we decided to use $NH_2OH \cdot HCl$ for the conversion of isatin to the corresponding isatinoxime as described earlier by Halket et al. [20]. Furthermore, the application of isatinoxime for calibration allowed the preparation of standards with *urine* instead of water thus comparable matrix effects were obtained for both calibration standards and urine samples. The use of a mixture of different urine samples ($n=5$) for preparation of the calibration solutions was necessary because we obtained a higher sensitivity (ca. threefold, data not shown) and increased peak areas for isatinoxime when the substance was dissolved in water instead of urine. The observed matrix effect might be due to interfering endogenous compounds that suppress the mass detector response but is likely also due to adsorption of the target compound to high molecular mass constituents of urine, e.g. peptides. Therefore we pooled urine samples of five different subjects to obtain comparable matrix effects for both calibration solutions and urine samples.

The derivatisation of the carbonyl group to the hydroxylamine function resulted in: (i) an increased retention at the precolumn and analytical column, (ii) a symmetrical peak shape, (iii) a higher mass detector response of isatinoxime compared to isatin, and (iv) the absence of interfering matrix compounds in the ion chromatograms of isatinoxime and the internal standard 5-methylisatinoxime. As shown in Fig. 3, the peak shape and sensitivity of isatinoxime and 5-methylisatinoxime are improved compared to their parent compounds.

The derivatisation of isatin with $NH_2OH \cdot HCl$ was found to be quantitative when the solution of isatin in water or urine was heated for 60 min at a temperature of 70°C. Although the reactivity of isatin allowed the derivatisation to the corresponding oxime at room temperature overnight as previously

described [20], we used 70°C to shorten the time for sample preparation. Since isatin was present in all urine samples studied (mean value 368 ng/ml) and 5-methylisatin is not a constituent of human urine, we used 5-methylisatin to prove the complete conversion of isatin in urine at various concentrations between 50 and 10 000 ng/ml (refer to Section 2.5.3). The resulting correlation coefficient ($R^2 = 0.9963$, $y = 0.0199x + 1.0269$) clearly indicates that there is no matrix effect on the derivatisation and that 5-methylisatin is fully converted to the corresponding 5-methylisatinoxime independent of its concentration.

The addition of $ZnSO_4$ to the urine samples after derivatisation resulted in a white precipitate obviously consisting of protein. This precipitation had no impact on recovery but improved the life time of the precolumns used for on-line sample extraction.

3.2. Validation

Recovery, inter-assay precision and accuracy experiments were performed using three quality control samples (50/750/4000 ng/ml). The average recovery ($n = 3$; mean values±standard deviation) of isatinoxime after $ZnSO_4$ precipitation and column switching was 94.9±10.9% (50 ng/ml), 73.3±2.2% (750 ng/ml) and 80.8±0.8% (4000 ng/ml). Since urine also contains lipophilic substances such as fatty acids and degradation products from bilirubin, the reduced recovery for the quality control samples with 750 and 4000 ng/ml isatinoxime might be the consequence of a reduced retention at the extraction column due to the higher affinity of lipophilic endogenous matrix compounds to the reversed-phase silica gel.

Linearity for isatinoxime was obtained from 5 to 5000 ng/ml ($R^2 = 0.9989$, $y = 0.0138x + 0.1328$) and the lower limit of detection was 2 ng/ml (signal-to-noise ratio 3). Inter-assay precision and accuracy for the quality control samples (Section 2.7.3; $n = 6$) were 1.8 and −1.5% (50 ng/ml), 1.9 and −4.9% (750 ng/ml), and 2.1 and +4.5% (4000 ng/ml). The concentration of the lowest quality control sample (50 ng/ml) was chosen following preliminary analyses of urine samples that most frequently exhibited a lowest concentration in the range of 50 ng/ml. However, due to the variation in isatin levels (refer to Table 2) we decided to use 5 ng/ml isatinoxime as the lowest calibration level. The herefore obtained precision was 6.1% ($n = 6$).

Table 2
Isatin levels and creatinine concentrations in urine samples of healthy humans

Urine sample	Isatin (ng/ml)	Creatinine (mg/ml)	ng Isatin/ mg creatinine
1	411	0.96	429
2	246	1.91	129
3	33	1.92	17
4	414	0.38	1095
5	672	1.59	424
6	131	1.64	80
7	804	0.57	1410
8	306	0.49	630
9	137	2.07	66
10	465	0.38	1214
11	322	1.29	249
12	471	3.14	150

3.3. LC/LC–MS

The automated on-line sample preparation step was preferable for the urine samples since the high salt content of urine does not allow direct injection onto the analytical column when MS detection is used. Furthermore, additional off-line sample preparation, such as solid-phase extraction or liquid–liquid extraction for purification and concentration, could be avoided. In order to prove the specificity of the assay described here, we analysed a blank urine sample obtained by pooling five different urine samples (Fig. 5A). No interfering matrix components are present at the retention time of isatinoxime (13 min; Fig. 5). Using selected ion monitoring at $m/z = 163$, isatinoxime can be clearly detected at a concentration of 5 ng/ml, which represents the lower limit of quantitation (Fig. 5B). Fig. 5 shows representative ion chromatograms obtained after analysis of a urine sample (No. 4, Table 2) containing 414 ng/ml isatinoxime (C) and 300 ng/ml of the internal standard 5-methylisatinoxime (D). The detection of the $[M+H]^+$ signals in the SIM mode at m/z 163 and m/z 177 provided excellent selectivity and sensitivity without the presence of interfering compounds.

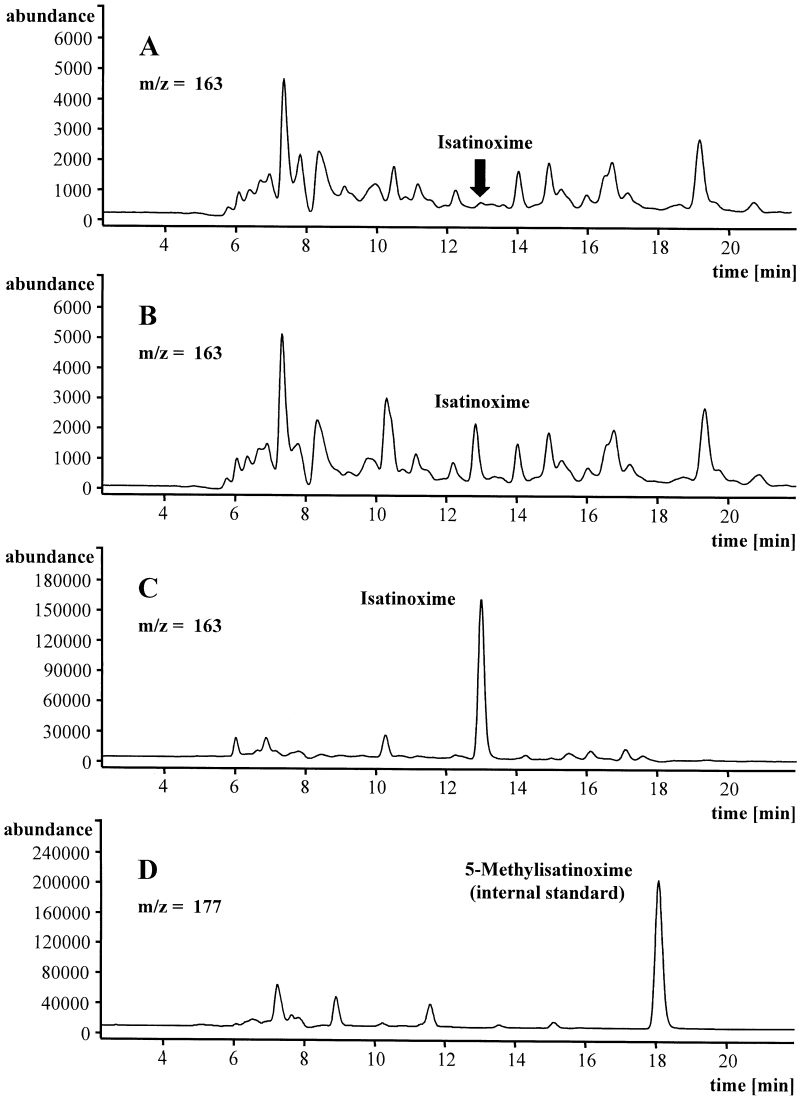

Fig. 5. Representative ion chromatograms for the determination of isatinoxime ($m/z=163$) and the internal standard 5-methylisatinoxime ($m/z=177$) in urine. (A) Extracted ion chromatogram ($m/z=163$) of a blank run (mixture of five different urine samples); (B) extracted ion chromatogram ($m/z=163$) of the pooled urine sample used in (A) spiked with 5 ng/ml isatinoxime; (C) extracted ion chromatogram ($m/z=163$) of urine sample No. 4 (Table 2); (D) extracted ion chromatogram ($m/z=177$) of urine sample No. 4 (for experimental details, refer to Sections 2.6 and 3.3).

3.4. Isatin levels in urine

Isatin was present in all urine samples studied. The mean value of isatin in urine was 368 ng/ml corresponding to 491 ng isatin/mg creatinine (Table 2) with no sex difference observed in the isatin levels (data not shown). The average level of isatin in urine determined by Manabe et al. [17] was 1342 ng isatin/mg creatinine (versus 491 ng isatin/mg creatinine, here) but the samples were heated for 10 min in a boiling water bath after adjustment of the pH value to 1 with 6 *M* HCl. This procedure might influence the isatin concentration because urinary indican (indoxylsulfate), the phase two metabolite of

indoxyl, is unstable as a free acid [23] and after hydrolysis and conversion to indoxyl is partially oxidised to isatin [24]. Since the oxidation of indoxyl to isatin is well known [25] and the indican level in urine of healthy humans is about 35 μg/ml [26] the conversion of indican to isatin could be a reasonable explanation for the higher isatin levels reported previously [17]. We observed marked individual variability concerning the isatin concentrations; for example the lowest isatin level was 17 ng/mg creatinine and the highest level obtained was 1410 ng/mg creatinine (Table 2). These interindividual differences in isatin levels are consistent with the data published by Manabe et al. [17].

At present, it is not known whether the isatin concentrations in urine reflect the amount of endogenous isatin produced via biosynthesis from amino acid precursors [27] or whether they represent the amount of dietary tryptophan converted to indole in the gut, absorbed and oxidised to isatin in the liver [28].

4. Conclusion

The quantitative determination of isatin in urine samples of healthy humans was performed using LC/LC–MS with column switching. After derivatisation of isatin with $NH_2OH \cdot HCl$ to isatinoxime and application of MS detection in the selected ion monitoring mode we obtained excellent sensitivity and selectivity, thereby avoiding a tedious and time consuming prepurification of urine samples. The method described here allows simple, fast and sensitive quantification of isatin in urine and is now routinely used in our laboratory to determine isatin in urine samples of patients suffering from renal diseases as will be reported subsequently. The direct injection of the urine samples after derivatisation following on-line sample extraction is an important prerequisite for clinical laboratories to analyse large amounts of urine samples without tedious and time consuming purification steps.

Acknowledgements

The financial support by the Lesmueller Foundation (Munich, Germany) for M.U. is greatly appreciated.

References

[1] W.E. Lambert, J.F. Van Bocxlaer, A.P. De Leenheer, J. Chromatogr. B 689 (1997) 45.
[2] T. Mizuma, L.Z. Benet, E.T. Lin, J. Chromatogr. B 718 (1998) 153.
[3] A.R. Jude, J.M. Little, J.P. Freeman, J.E. Evans, A. Radominska-Pandya, D.F. Grant, Arch. Biochem. Biophys. 380 (2000) 294.
[4] F. Baumann, C. Lorenz, U. Jaehde, R. Preiss, J. Chromatogr. B 729 (1999) 297.
[5] N. Serkova, B. Hausen, G.J. Berry, W. Jacobsen, L.Z. Benet, R.E. Morris, U. Christians, J. Pharmacol. Exp. Ther. 294 (2000) 323.
[6] U. Timm, H. Birnboeck, R. Erdin, G. Hopfgartner, R. Zumbrunnen, J. Pharm. Biomed. Anal. 21 (1999) 151.
[7] F. Guan, A. Ishii, H. Seno, K. Watanabe-Suzuki, T. Kumazawa, O. Suzuki, Anal. Chem. 71 (1999) 4034.
[8] S.T. Wu, Y.P. Chang, W.L. Gee, L.Z. Benet, E.T. Lin, J. Chromatogr. B 692 (1997) 133.
[9] R. Turci, M.L. Fiorentino, C. Sottani, C. Minoia, Rapid Commun. Mass Spectrom. 14 (2000) 173.
[10] A. Ramaiya, T. Karnes, J. Chromatogr. B 691 (1997) 119.
[11] U. Christians, W. Jacobsen, N. Serkova, L.Z. Benet, C. Vidal, K.F. Sewing, M.P. Manns, G.I. Kirchner, J. Chromatogr. B 748 (2000) 41.
[12] S.E. Nielsen, R. Freese, C. Cornett, L.O. Dragsted, Anal. Chem. 72 (2000) 1503.
[13] M. Sandler, A. Clow, P.J. Watkins, V. Glover, Stress Med. 4 (1988) 215.
[14] V. Glover, J.M. Halket, P.J. Watkins, A. Clow, B.L. Goodwin, M. Sandler, J. Neurochem. 51 (1988) 656.
[15] P.J. Watkins, A. Clow, V. Glover, J.M. Halket, A. Przyborowska, M. Sandler, Neurochem. Int. 17 (1990) 321.
[16] V. Glover, Biog. Amines 9 (1993) 443.
[17] S. Manabe, Q. Gao, J. Yuan, T. Takahashi, A. Ueki, J. Chromatogr. B 691 (1997) 197.
[18] Y. Tozawa, A. Ueki, S. Manabe, K. Matsushima, Biochem. Pharmacol. 56 (1998) 1041.
[19] F.Y. Pang, F.H. Hucklebridge, G. Forster, K. Tan, A. Clow, Stress Med. 12 (1996) 35.
[20] J.M. Halket, P.J. Watkins, A. Przyborowska, B.L. Goodwin, A. Clow, V. Glover, M. Sandler, J. Chromatogr. A 562 (1991) 279.
[21] N. Hamaue, N. Yamazaki, M. Minami, T. Endo, M. Hirahuji, Y. Monma, H. Togashi, Gen. Pharmacol. 30 (1998) 387.
[22] M. Jaffé, Z. Physiol. Chem. 10 (1886) 391.
[23] The Merck Index, Vol. 12, Merck and Co., Inc., Whitehouse Station, NJ, 1996, p. 849.
[24] E.M.J. Gillam, L.M. Notley, H. Cai, J.J. De Voss, F.P. Guengerich, Biochemistry 39 (2000) 13817.
[25] H. Bialy, Nat. Biotechnol. 15 (1997) 110.
[26] J.L. Kirkland, M. Lye, E. Vargas, Postgrad. Med. J. 59 (1983) 717.
[27] A.E. Medvedev, A. Clow, M. Sandler, V. Glover, Biochem. Pharmacol. 52 (1996) 385.
[28] M. Sandler, A. Przyborowska, J.M. Halket, P. Watkins, V. Glover, M.E. Coates, J. Neurochem. 57 (1991) 1074.