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Comparison of Procedures for Measuring the Quaternary *N*-Glucuronides of Amitriptyline and Diphenhydramine in Human Urine with and without Hydrolysis

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Abstract

The activities of β-glucuronidases from *Helix pomatia*, *Escherichia coli* and rat towards the *N*-glucuronides of amitriptyline and diphenhydramine were considerably lower than those towards standard substrates.

The two *N*-glucuronides were analysed in urine samples by the following procedures: HPLC of the intact conjugate after solid-phase extraction on a cation exchanger cartridge or after direct injection of urine; HPLC of the aglycone after hydrolysis with β-glucuronidase from *H. pomatia* or *E. coli* or after alkaline hydrolysis. Solid-phase extraction led to the highest recovery and precision, and sensitivity can be improved by extracting a larger volume of urine.

On application to samples from patients under treatment with amitriptyline, the results of all procedures except alkaline hydrolysis were in good agreement. When diphenhydramine *N*-glucuronide was analysed in urine samples of volunteers, solid-phase extraction, hydrolysis by *E. coli* glucuronidase and alkaline hydrolysis resulted in similar values.

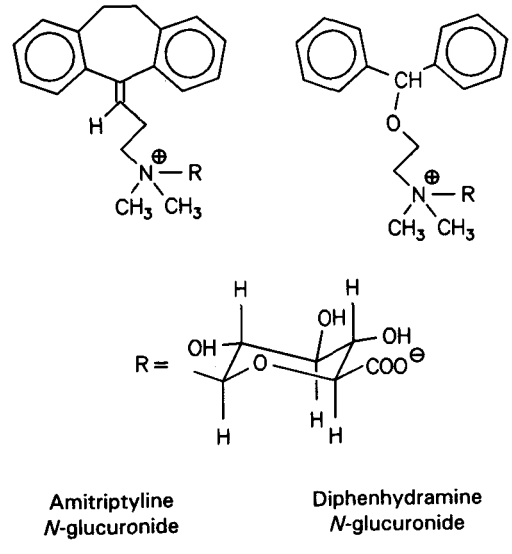
Conjugation of tertiary amino groups with glucuronic acid to form quaternary ammonium glucuronides may make a significant contribution to the metabolism of basic lipophilic drugs in man. This has been shown for aliphatic and saturated heterocyclic amines such as cyproheptadine (Porter et al 1975; Fischer et al 1980), cyclobenzaprine (Hucker et al 1978), various antihistamines (Chaudhuri et al 1976; Luo et al 1991a) and tricyclic antidepressants (Breyer-Pfaff et al 1978; Vandel et al 1982; Lehman et al 1983; Luo et al 1991b), as well as for drugs with unsaturated heterocyclic rings (for instance Macrae et al 1990; Sinz & Remmel 1991). Isolation of the intact conjugates served for structure assignment, and this procedure as well as hydrolysis to the aglycone was used for quantification in a few subjects. Measurements in groups of patients or volunteers seem to have been confined to amitriptyline *N*-glucuronide (Fig. 1) that was analysed either as the intact compound by HPLC (Dahl-Puustinen et al 1989; Breyer-Pfaff et al 1990, 1992) or as amitriptyline following hydrolysis by enzymes (Vandel et al 1982) or alkali (Dahl-Puustinen et al 1989).

The aim of the present investigation was to evaluate previously described procedures for amitriptyline *N*-glucuronide determination and to establish a sensitive and selective procedure that can be applied for analysing this conjugate and other quaternary ammonium glucuronides. As an example of the latter, diphenhydramine *N*-glucuronide (Fig. 1) has been chosen.

Materials and Methods

Substances

Amitriptyline hydrochloride was kindly donated by Troponwerke (Köln, Germany). Its *N*-glucuronide was isolated in milligram quantities from human urine (Breyer-Pfaff et al 1990). In addition, it was synthesized by the reaction of amitriptyline with methyl-1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranosuronate according to the method of Luo et al (1992), with the following modifications: the glucuronic acid derivative was dissolved in toluene, the mixture was stirred for seven days instead of three days, and the product was isolated from the washed aqueous layer by adsorption onto C_{18}-silica (6 mL, Polygosil, 40–63 μm, Macherey-Nagel, Düren, Germany). After washing with water, the conjugate was eluted with methanol and purified by thin-layer chromatography on silica gel with fluorescent



Amitriptyline *N*-glucuronide

Diphenhydramine *N*-glucuronide

FIG. 1. Structural formulas of amitriptyline and diphenhydramine *N*-glucuronides.

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indicator in *n*-butanol : acetic acid : water (4 : 1 : 1, v/v). The UV-absorbing band (R_f 0·47) was eluted with water and the glucuronide isolated by adsorption on C_{18}-silica as above. From diphenhydramine hydrochloride (Sigma, St Louis, USA), the *N*-glucuronide was prepared by the same procedure. Its R_f value in thin-layer chromatography was 0·36.

Since these *N*-glucuronides did not crystallize, their purities were determined by weighing and by measuring the UV absorption of aliquots diluted in water. With the assumption that the molar absorption differences at two wavelengths of the *N*-glucuronides are the same as those of the parent compounds, synthetic amitriptyline *N*-glucuronide had a purity of 90% and diphenhydramine *N*-glucuronide of 97·5%. These values were used to adjust quantities taken for standardization or added to urine in recovery experiments. No additional peaks appeared on HPLC of any preparation, justifying this approach.

β-Glucuronidase from *Escherichia coli* K 12 and β-glucuronidase/arylsulphatase from *Helix pomatia* were purchased from Boehringer Mannheim. A preparation of β-glucuronidase was obtained from rat preputial glands and purified up to the stage of Sephadex G 200 filtration (Tulsiani & Touster 1978).

Urine samples

Patients under treatment with amitriptyline hydrochloride, 150 mg per day, collected 24-h urine that was stored at −20°C. Some of the patients were also being treated with oxazepam, clorazepate or flunitrazepam. Two volunteers ingested commercial tablets containing 25 or 50 mg diphenhydramine hydrochloride and collected 24-h urine in 8-h fractions.

Specific activities of glucuronidases towards N-*glucuronides*

As reference values, the activities of the enzyme preparations were determined using phenolphthalein glucuronide as substrate (Stahl & Fishman 1984). With the enzyme mixture from *H. pomatia*, the glucuronidase activity was found to be 7 units mL^{-1} at 37°C (supplier information: ca 5·5 units mL^{-1}). Hydrolyses of amitriptyline *N*-glucuronide were carried out in 1 mL 0·1 M sodium acetate buffer, pH 5·0, containing 167 nmol substrate and 2·5, 5 or 10 μL enzyme solution in incubations at 56°C (the temperature used by Vandel et al (1982)) and 5, 10 or 20 μL in samples incubated at 37°C. Portions (0·25 mL) were removed after 2, 4 and 6 h and acidified with 0·025 mL 0·5 M sulphuric acid. Amitriptyline was determined by injecting 0·25 mL for HPLC (see below). Incubations with the enzyme preparation from rat were carried out in the same manner. For hydrolyses with β-glucuronidase from *E. coli*, 75 mM potassium phosphate buffer, pH 6·8, was used, and an activity of 2·5 units mL^{-1} towards phenolphthalein glucuronide was found at 37°C (supplier information: 200 units mL^{-1} with 4-nitrophenyl glucuronide as substrate). Samples containing 134 nmol amitriptyline *N*-glucuronide and 1–4 μL enzyme in 1 mL buffer were incubated at 37°C and after 0·5, 1 and 2 h, portions (0·25 mL) were removed and at once acidified for subsequent HPLC.

When diphenhydramine *N*-glucuronide was the substrate, 175 nmol was incubated with 2, 4 or 8 μL β-glucuronidase (+arylsulphatase) from *H. pomatia* in 1 mL 0·1 M sodium acetate buffer, pH 5·0, for 1–3 h at 56°C. The enzyme from *E. coli* (1 or 2 μL) was tested towards 137 nmol of conjugate at pH 6·8 during 0·5, 1 and 2 h at 37°C. Acidified portions (0·25 mL) could be analysed directly by HPLC.

Enzymatic hydrolysis for N-*glucuronide quantification*

Incubations were performed in a similar manner as above, but an excess of enzyme was used to achieve complete hydrolysis. Urine samples (3 mL) from patients were adjusted to pH 9 with 25% ammonia and unconjugated drug and metabolites extracted with 2 mL of chloroform. For incubations with the enzyme from *H. pomatia*, the urine was adjusted to pH 5 with 1 M acetic acid and 0·4 mL was mixed with 0·1 mL β-glucuronidase/arylsulphatase and 0·05 mL 1 M sodium acetate, pH 5·0. After 12 h at 56°C, samples were cooled, adjusted to pH 9 with ammonia and after addition of 150 mg NaCl and 0·06 mL 10% (w/v) sodium deoxycholate extracted twice with 0·5 mL *tert*-butyl methyl ether. The combined organic phases were extracted with 0·35 mL 0·05 M H_2SO_4, the acid layer was washed with 0·5 mL *n*-hexane and 0·25 mL was injected for HPLC. Samples of control urine containing added amitriptyline or diphenhydramine *N*-glucuronide were treated in the same manner. Quantities recovered could not be increased by increasing the amount of enzyme or the incubation time. The stability of amitriptyline and the quality of the extraction procedure were tested by adding amitriptyline to incubations of control urine.

For hydrolysis with *E. coli* glucuronidase, extracted urine samples (0·4 mL) were adjusted to pH 6·8 with phosphoric acid, buffered with 0·05 mL 0·75 M potassium phosphate, pH 6·8, and incubated for 12 h at 37°C with 0·02 or 0·015 mL enzyme for the hydrolysis of amitriptyline or diphenhydramine *N*-glucuronide, respectively. Extraction of liberated aglycones was performed as described above.

β-Glucuronidase from rat was not used for quantitative hydrolysis because of its low activity towards amitriptyline *N*-glucuronide and its limited supply.

Alkaline hydrolysis of N-*glucuronides*

Urine samples pre-extracted with chloroform or *tert*-butyl methyl ether were incubated according to Dahl-Puustinen et al (1989) with NaOH at a final concentration of 0·67 M for 15 min at 100°C, and the liberated drugs were extracted by the procedure used after enzymatic hydrolysis. The yield could not be increased by using a higher NaOH concentration or a longer incubation time.

Solid-phase extraction of N-*glucuronides*

The cartridges (SCX 500 mg, Analytichem International, Harbor City, USA) contained sulphonyl-phenylpropyl-silica, a strongly acidic cation exchanger. They were conditioned with 3 mL 1 M acetic acid and 3 mL water and after application of urine (usually 1 mL diluted with 2 mL water) washed with 2 mL methanol. Elution was with 6 mL 0·2 M ammonium acetate : methanol : acetonitrile (1 : 2 : 8, v/v) that was evaporated completely at 40°C under nitrogen. The residue was dissolved in 0·4–1 mL 0·05 M H_2SO_4 of which 0·25 mL was used for HPLC. Cartridges with 100 mg sorbent were used for urine quantities up to 0·3 mL. They could be conditioned with 1 M HCl (the 500 mg cartridges

Table 1. Relative activities of β-glucuronidases from various species in hydrolysing the quaternary ammonium glucuronides of amitriptyline and diphenhydramine. Values given are the activities towards these substrates of 1 unit of the enzymes determined at 37°C with phenolphthalein glucuronide as substrate.

Substrate	Temperature (°C)	Activity (m units) of enzyme		
		Helix pomatia	*Escherichia coli**	Rat
Amitriptyline *N*-glucuronide	37	0·4–1	56	0·4–1
	56	1·2–2		3·4–4·7
Diphenhydramine *N*-glucuronide	37	—	210	
	56	2·1–4		

* The enzyme quantity corresponds to about 80 units with *p*-nitrophenyl glucuronide as substrate.

were unstable towards HCl) and elution was with 0·1 M instead of 0·2 M ammonium acetate.

HPLC

Samples were applied to a pre-column of 6 × 5 mm that contained C_2-silica (Sepralyte, 40 μm, Analytichem) in analyses of amitriptyline or its conjugate and C_{18}-silica (Polygosil) for diphenyhydramine or its conjugate. After washing with water (1·5 mL min^{-1}) for 3 min, the pre-column was eluted with the solvent in reverse direction onto a column of 4·6 × 250 mm with C_{18}-silica (Nucleosil 5 μm, Macherey-Nagel). The solvent was 10 mM perchloric acid adjusted to pH 2·5:acetonitrile (70 : 30, v/v, for amitriptyline *N*-glucuronide, retention time R_T 13·8 min, 60 : 40 for amitriptyline, R_T 13·0 min, 71 : 29 for diphenhydramine *N*-glucuronide, R_T 11·8 min, 62 : 38 for diphenylhydramine, R_T 10·6 min), flow rate 1·2 mL min^{-1}. The eluate was monitored for UV absorption at 254 nm for amitriptyline and its glucuronide and at 220 nm for diphenhydramine, and its glucuronide. Before injection of the next sample, the pre-column was conditioned with water.

Quantification was based on peak heights measured by an integrator in relation to those of external standards diluted in 0·05 M sulphuric acid. Calibration lines comprising the range of quantities present in the samples were linear and passed through the origin. Recoveries were calculated as the ratios of nmol *N*-glucuronide or aglycone found to nmol of synthetic *N*-glucuronide added to control urine samples, multiplied by 100.

For determination of amitriptyline *N*-glucuronide by direct injection to HPLC, 0·9 mL urine was acidified with 0·1 mL 0·5 M H_2SO_4 and 0·25 mL was injected. Since oxazepam contained in some of the patient samples interfered with amitriptyline *N*-glucuronide in the above solvent, separation was achieved with 0·02 M ammonium acetate (adjusted to pH 5: methanol) (50 : 50, R_T amitriptyline *N*-glucuronide 18 min, R_T oxazepam 20 min at a flow rate of 1 mL min^{-1}).

Results

The quaternary ammonium glucuronides of amitriptyline and diphenhydramine proved to be poorer substrates of the three β-glucuronidases from different species than phenolphthalein glucuronide. With the enzyme from *H. pomatia*, the hydrolysis rate declined with time with both substrates and under various conditions of temperature and enzyme : substrate ratio. The same applied to the reaction of amitriptyline *N*-glucuronide with β-glucuronidase from rat. Therefore, ranges of activities are given for these enzymes in Table 1. Instability of the enzyme does not seem to be responsible, because incubation of *H. pomatia* enzyme for up to 24 h at 56°C did not lead to a loss of activity towards phenolphthalein glucuronide. In incubations with *E. coli* β-glucuronidase, liberation of aglycones was linear with time. This enzyme exhibited the highest activity towards amitriptyline and diphenhydramine *N*-glucuronides relative to phenolphthalein glucuronide.

Results of recovery experiments on control urine samples with various quantities of the *N*-glucuronides added are summarized in Table 2. Analysis of the intact conjugates after extraction on cartridges with 500 mg of a silica-bonded strongly acidic cation exchanger (SCX) led to high recoveries with good precision. Equally good recoveries were observed when small volumes of control urine with added amitriptyline *N*-glucuronide were extracted on cartridges with 100 mg SCX. No interfering peaks appeared on chro-

Table 2. Recoveries of *N*-glucuronides (4–25 nmol mL^{-1}) after addition to control urine and analysis by procedures with or without hydrolysis.

Procedure	Recovery (%) of added *N*-glucuronides	
	Amitriptyline	Diphenhydramine
Without hydrolysis		
Extraction on SCX cartridges 500 mg	93 ± 5 (17)	92 ± 4 (11)
100 mg	94 ± 5 (22)	
With hydrolysis by		
β-Glucuronidase from *Helix pomatia*	81 ± 7 (11)	83 ± 1 (8)
Escherichia coli	85 ± 8 (11)	91 ± 9 (8)
NaOH 0·67 M 100°C	38 ± 9 (12)	73 ± 6 (6)

Values are means ± s.d., numbers of determinations are given in parentheses.

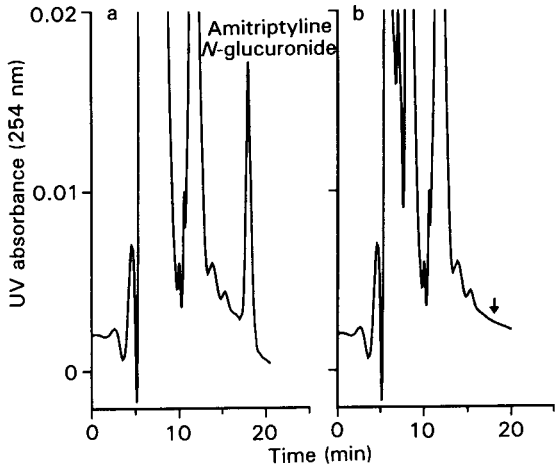

FIG. 2. HPLC of extracts (SCX cartridges 500 mg) from the urine of a patient receiving 150 mg amitriptyline per day. a, 1 mL native urine was extracted and one-fourth, corresponding to 3·4 nmol N-glucuronide, was injected; b, extraction was after hydrolysis of 1 mL urine with 0·015 mL *E. coli* glucuronidase for 2 h at 37°C and an aliquot corresponding to 0·25 mL urine was injected (the arrow indicates the retention time of the glucuronide).

matograms of control urine extracts. The procedure has the further advantage that in the case of low concentrations, its sensitivity can be increased by processing larger volumes. Samples of 10 mL control urine with added amitriptyline N-glucuronide at 0·5 or 1 nmol mL^{-1} were extracted on 500 mg-cartridges and the recoveries were equally good as with higher concentrations (Table 2). Diphenyhydramine N-glucuronides concentrations down to 1·3 nmol mL^{-1} could be measured in urine volumes of 1–3 mL without a decrease of recovery, whereas losses occurred with larger volumes. Direct injection for HPLC of acidified control urine samples with added amitriptyline conjugate resulted in the same peak heights as when standard solutions diluted with acid were injected. The storage stability at −20°C was tested by re-analysing urine from volunteers after nine months. In five samples, the diphenyhydramine N-glucuronide quantities found were 100 ± 8% of those measured before storage.

Of the procedures tested for liberating the aglycones, cleavage with β-glucuronidase from *E. coli* resulted in the highest recoveries, while somewhat lower values were achieved with that from *H. pomatia*. Recoveries were no greater when amitriptyline N-glucuronide was added to water instead of to urine. The failure to attain 100% recovery was apparently not due to a loss of liberated aglycone, because amitriptyline added to an incubation mixture with *H. pomatia* enzyme was completely recovered (99 ± 4%, n = 6). The poorest results were obtained when deconjugation was attempted by heating with alkali. This applied particularly to amitriptyline conjugate from which on average less than 40% of the calculated amitriptyline quantity could be recovered. The mean of 73% diphenhydramine obtained from its glucuronide was markedly better, but still inferior to the results of enzymatic hydrolysis (Table 2).

The various procedures were applied to urine samples from amitriptyline-treated patients. The specificity of the solid-phase extraction with subsequent HPLC was tested by analysing urine before and after treatment with *E. coli* glucuronidase. The representative example in Fig. 2 shows that after deconjugation of the amitriptyline conjugate, no interfering peak appeared in the chromatogram.

When the concentrations of amitriptyline N-glucuronide measured in seven patient urine samples by extracting on 500 mg SCX are taken as 100%, values obtained by the other procedures and corrected for the recoveries shown in Table 2 were (means ± s.d.): extraction on 100 mg SCX 98 ± 8%, direct injection 108 ± 13%, hydrolysis with β-glucuronidase from *H. pomatia* or *E. coli* 100 ± 18 and 94 ± 21%, respectively, and alkaline hydrolysis 146 ± 25%. Thus, the latter method only gave results clearly deviating from those expected on the basis of the recovery experiments. The various procedures were also compared with regard to CV in duplicate determinations. These varied from 4 and 5% for the extractions on 500 mg and 100 mg SCX to 18% for alkaline hydrolysis, while enzymatic hydrolysis resulted in intermediate CV values of 10% with the *H. pomatia* and 6% with the *E. coli* enzyme. The same comparison was carried out for N-glucuronide analyses in eight urine samples collected after diphenyhydramine ingestion. Relative to extraction on 500 mg SCX, hydrolysis with *E. coli* glucuronidase and NaOH resulted in values of 107 ± 12 and 96 ± 16%; CV values were 8, 10 and 17%, respectively.

Discussion

Previous procedures for the isolation of quaternary ammonium glucuronides present as drug metabolites in urine included adsorption to a nonionic polymer (XAD-2), sometimes followed by chromatography on a weakly basic anion exchanger (Chaudhuri et al 1976; Lehman et al 1983; Macrae et al 1990; Luo et al 1991a, b; Sinz & Remmel 1991). Adsorption to a cation exchanger was one of three steps in the purification of cyproheptadine N-glucuronide (Porter et al 1975). The present results demonstrate that the positive charge of quaternary N-glucuronides may be used for nearly complete recovery and efficient purification of these metabolites from urine by adsorption to a cation exchanger before their quantification by HPLC. A high sensitivity can be achieved by extracting large volumes of urine. The limit of quantification for amitriptyline N-glucuronide is below 0·5 nmol mL^{-1}, the lowest concentration investigated. The specificity on analysing urine from amitriptyline-treated patients was confirmed by the absence of a peak in HPLC of enzymatically hydrolysed samples (Fig. 2). The good accordance between values measured in patient samples without and with enzymatic deconjugation demonstrates the accuracy of the solid-phase extraction-HPLC method. The same applies to the determination of diphenhydramine N-glucuronide in the urine of volunteers. With regard to reproducibility in amitriptyline N-glucuronide analyses, extraction on SCX cartridges proved superior to the procedures using deconjugation, while for the determination of diphenhydramine conjugate, it was at least as good.

Unconjugated oxazepam that may be present in urine of patients co-medicated with this drug or one of its metabolic precursors co-chromatographed with amitriptyline N-glucuronide in perchloric acid pH 2·5: acetonitrile (70 : 30).

Whereas it is removed by solid-phase extraction, it can produce elevated values on direct injection of patient urine for HPLC (Breyer-Pfaff et al 1992).

As an alternative to measurement of the intact *N*-glucuronide, enzymatic liberation and subsequent quantification of the parent drug has been applied for determining amitriptyline *N*-glucuronide in urine (Breyer-Pfaff et al 1978; Vandel et al 1982). Using this procedure, the relative resistance of the conjugate towards hydrolysis by the commonly-used preparation of β-glucuronidase/arylsulphatase from *H. pomatia* needs to be considered. This enzyme deconjugated amitriptyline *N*-glucuronide at a rate that was not more than 1/500th that with phenolphthalein glucuronide, and large quantities of enzyme have to be added to urine to achieve near-complete hydrolysis. Therefore, the better recovery of amitriptyline after alkali than after enzyme treatment of patient urine has probably been due to the small amount of enzyme added (Dahl-Puustinen & Bertilsson 1987). The activity of β-glucuronidase from *E. coli* towards the *N*-glucuronides also was at most 1/200th that towards its standard substrate *p*-nitrophenyl glucuronide. With sufficient quantities of enzyme, mean recoveries of aglycones were 85% from amitriptyline *N*-glucuronide and 91% from diphenhydramine *N*-glucuronide and thus slightly exceeded those achieved with the *H. pomatia* enzyme preparation.

Alkaline hydrolysis has been observed with the *N*-glucuronides of tripelennamine (Chaudhuri et al 1976) and amitriptyline (Dahl-Puustinen & Bertilsson 1987), and it has been used for quantification of amitryptyline *N*-glucuronide (Dahl-Puustinen et al 1989). In the present experiments, mean amitriptyline recoveries were markedly lower (38%) on alkaline than on enzymatic hydrolysis (91% with *E. coli* β-glucuronidase) when conjugate had been added to control urine. In analyses of patient urine, the discrepancy still existed, but it was less: 56% with NaOH vs 86% with *E. coli* enzyme of the values found with solid-phase extraction (without correcting for recovery). This points to the possibility that amitriptyline or a compound co-chromatographing with it was formed from a metabolite other than the *N*-glucuronide. Amitriptyline *N*-oxide is partially converted to amitriptyline under the conditions of alkaline hydrolysis, but this metabolite is largely removed when urine is extracted with chloroform before heating with alkali. Diphenhydramine *N*-glucuronide added to blank urine was 73% hydrolysed on heating with NaOH, and comparison with solid-phase extraction and enzymatic hydrolysis points to a similar percentage in urine samples of volunteers. However, the precision was poorer and also with regard to sensitivity and simplicity, sample preparation by extraction on a cation exchanger seems preferable. In conclusion, this latter procedure in conjunction with HPLC can be recommended for the analysis of the *N*-glucuronides investigated and probably also of related compounds.

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