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Review

Glucuronic acid conjugates

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Abstract

The methods of assay in body fluids of 1-β-alkyl, 1-β-phenyl and 1-β-acyl glucuronic acids (''glucuronide conjugates'') have been reviewed. Most of the 78 references cited (from the literature of the period 1990–1997) concern the glucuronide conjugates of drug metabolites, and these have been considered, for reasons of accessibility, within sections of individual drug classes such as analgesics, anti-cancer agents and opioids. Other glucuronide conjugates are considered under ''miscellaneous compounds''. A few gas chromatography and capillary electrophoresis methods are described, but the major technique of assay (62 citations) is reversed-phase high-performance liquid chromatography.

Keywords: Reviews; Glucuronic acid

Contents

1. Introduction

The glucuronic acid conjugates, or ''glucuronide conjugates'' as they are commonly called, are compounds formed by enzyme-catalysed glucuronidation of drug metabolites that contain either a hydroxyl group (ROH – alcohols; PhOH – phenols) or a carboxyl group (RCO_2H – acids). The glucuronida-

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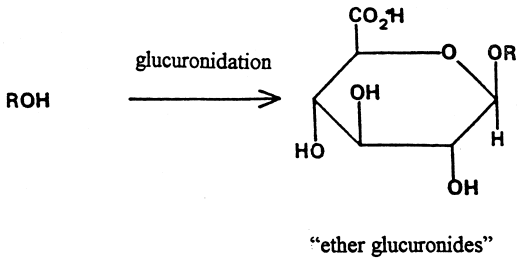
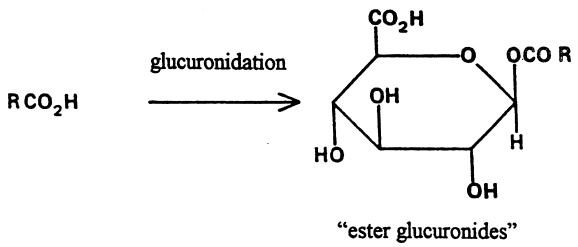


Scheme 1.

tion process involves the attachment to the metabolite, at its hydroxyl group (Scheme 1) or its carboxyl group (Scheme 2), of a glucuronic acid residue, the join being at the β-1 position of the glucuronic acid.

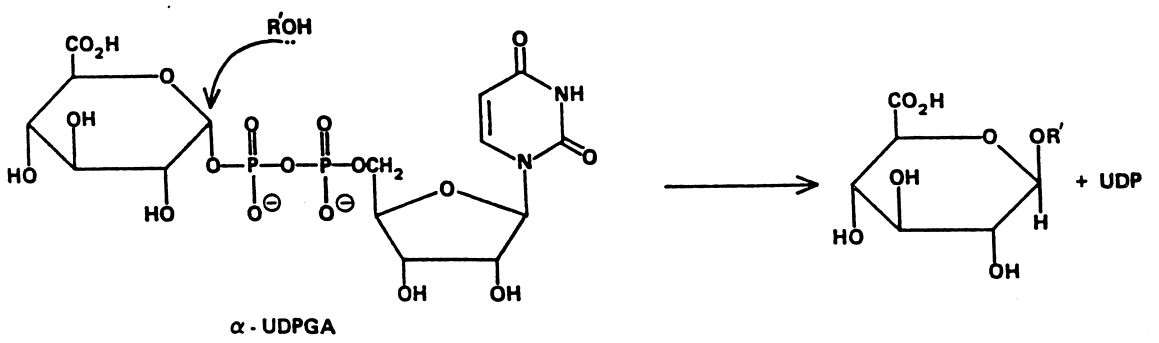
A coenzyme, uridine 5′-diphospho-α-D-glucuronic acid, provides the glucuronic acid moiety and the class of enzyme that catalyses the process is a glucuronyltransferase. To all intents and purposes, the enzyme catalyses a nucleophilic S_N2 displacement reaction – which proceeds with inversion of configuration at C_1, since the α-1 uridine diphosphate group is replaced by a β-1 alkoxyl or phenoxy group (Scheme 3, R′=alkyl or phenyl) or a β-1 acyloxy group (Scheme 3, R′=acyl). The products of the reaction are best known, respectively, as ''ether glucuronides'' or ''ester glucuronides''. Their systematic names are, respectively, 1-β-alkyl (or 1-β-phenyl) α-D-glucuronides and 1-β-acyl α-D-glucuronides. Strictly, the latter, as esters, are systematically named as 1-β-D-glucuronyl carboxylates. Thus, the systematic name of the ''ester glucuronide'' of benzoic acid is 1-β-D-glucuronyl benzoate. In general, in this review the author has, for convenience, used the names employed by the authors of the cited papers, rather than change all of them to the authentic systematic names.



Scheme 2.

To the author's knowledge, this is the first review of the quantitative analysis of glucuronide conjugates. The reason for this is that it is only since the advent of reversed-phase high-performance liquid chromatography (RP-HPLC) that quantitation of analytes as polar as glucuronide conjugates has been achievable. This technique was being widely used for the analysis of many other types of polar compounds in the early 1980s, yet its application to the assay of glucuronide conjugates did not become widespread until the early 1990s.

The reason for that delay is, in large part, due to the ready commercial availability of glucuronidases, the enzymes that hydrolyse glucuronide conjugates. A common protocol adopted by the drug metabolism fraternity, until recently, was to incubate ''polar'' drug metabolites suspected of being glucuronide conjugates with, say, *E. coli* glucuronidase and then to quantify the released ''aglycone''. In this way, an estimate of the extent of formation of a particular metabolite from a drug was possible by aggregating



Scheme 3.

the amount present in excreta of the ''free'' metabolite and that of the ''enzyme-released'' metabolite derived from the conjugate. These data, in general, sufficed for ''fate'' studies of drugs, where knowledge only of percentage unchanged drug and percentage metabolised drug were required.

It has always been important to elucidate the structure of the ''free'' (non-conjugated) metabolites – and much effort has been devoted to this, but rarely was there seen to be a need to characterise an intact glucuronide conjugate – let alone develop an assay for it! The major reason for this was that they were regarded as ''detoxified'' drug metabolites; as, in most cases they were, since they were readily excreted – often as ''water-soluble'' metabolites via the kidney. However, several examples of biologically-active or toxic glucuronide conjugates of drugs came to light, and it was clearly an oversimplification to regard all glucuronide conjugates as detoxification products. A celebrated example is morphine-6-glucuronide which shows morphine-like activity in man. That (belated) recognition of the importance of glucuronide conjugates per se led to a surge of interest in their identification and quantitation. This began in the late 1980s and has reached its maturity in the late 1990s. Thus a review is timely.

The major technique for the determination of glucuronide conjugates, not surprisingly, is HPLC. Indeed, about 80% of the 78 references cited in this review are concerned with HPLC methods, with just a handful devoted to gas chromatography (GC) and electrophoretic methods. The recent introduction of capillary electrophoresis (CE), which allows quantitation of polar compounds, has provoked some interest latterly, however. An attractive feature of CE is that the clean-up of body fluid samples is simple, or can be dispensed with.

This review has largely concentrated on the literature of the late 1990s, with more than half of the cited references falling in the period 1995–1997. The aim has been for a comprehensive, but not an exhaustive, coverage of the literature. As can be seen from the list of contents, there are sections of the review devoted to drugs of different classes (e.g., analgesics, opioids) and to miscellaneous chemicals, grouped under headings entitled *O*-containing, *N*-containing and halogen-containing compounds. This review is going to press in early 1998, and this has meant that the literature coverage is virtually complete up to the end of 1997.

2. Chromatographic methods

2.1. High-performance liquid chromatography

2.1.1. General methodology

A mixed-bed HPLC stationary phase of equal parts of C_{18} phase and of cationic-exchange phase has been evaluated as a means of expeditiously analysing samples containing (charged) glucuronides and their (uncharged) parent compounds. The test compounds were phenyl and 4-nitrophenyl glucuronides, and the methodology was shown to have good potential [1].

2.1.2. Drugs

2.1.2.1. *Benzodiazepines*. A rapid and simple method for the simultaneous detection and quantitation of diastereoisomeric benzodiazopine glucuronides has been described [2]. Among those investigated were oxazepam, temazepam and lorezepam.

Oxazepam, which is an active metabolite of various benzodiazepines, is extensively converted in vivo into its glucuronide. Since the C_3 position of oxazepam is chiral, there are two enantiomeric forms of oxazepam and these give rise to two diastereoisomeric glucuronides, *S*(+) and *R*(−), the ratios of each being species-dependent. The quantification of each enantiomer has been accomplished by direct injection of diluted acidified (pH 2) sheep urine onto a reversed-phase column [3]. The same group of workers has developed a method for assaying these compounds in sheep plasma, by use of a clean-up by solid-phase extraction (SPE) using a C_{18} cartridge [4].

2.1.2.2. *Analgesics*. The acyl glucuronides of naproxen and 6-*O*-demethylnaproxen can be assayed simultaneously by gradient elution RP-HPLC with UV detection [5]. Glucuronides of indomethacin and its metabolites have been determined in human plasma and urine by means of a direct gradient system using a C_8 column [6]. The glucuronide of phenazone in urine was assayed by extraction and

derivatization by methylation (diazomethane) and acetylation [acetic anhydride (Ac_2O)/pyridine] followed by isocratic RP-HPLC [7].

Using a C_8 and a C_{18} column in series, it has been possible to resolve the phenyl and the acid glucuronides of salicylate, the primary aspirin metabolite. The glycine conjugate of salicylate was also resolved and determined [8]. The simultaneous determination in urine of paracetamol, its glucuronide and its sulfate has been achieved by RP-HPLC using UV detection [9].

The readily formed acyl glucuronide of ibuprofen has been determined in human plasma using a C_{18} column [10]. Ketoprofen, 2-(3-benzoylphenyl)propanoic acid (Profenid), is used therapeutically as its racemate. Biosynthesis of the pair of diastereoisomeric ketoprofen acyl glucuronides could be effected with rat liver microsomes. The simultaneous analysis of each of the pair was achieved using ion-pair RP-HPLC at pH 4.3 – the pH being crucial to the success of the assay [11]. Another group [12] also reported the resolution of the two ketoprofen acyl glucuronides, but was unable to assay the intact compounds in plasma because of matrix interferences.

2.1.2.3. *Opioids*. There are several reports on the serious topic of morphine glucuronide assay. Two glucuronides are important, morphine-6- (M6G) and morphine-3-glucuronide (M3G), the former especially so since it shows high biological activity and does not constitute (as a glucuronide often does) a detoxication product. The main application of the methods is testing for drugs of abuse, and the emphasis is on methods which will provide a simultaneous assay of morphine and its glucuronides, thereby giving an indication of total dose.

In 1991, HPLC with multi-wavelength forward optical detection was used to determine morphine and its glucuronides in biological fluids. An impressive limit of detection of 500 pg ml^{-1} was achieved by Gulati et al. [13].

Zweipfennig [14] has raised doubts about the limits of detection claimed, and Gulati [15] has replied. Further doubt was voiced by Bogusz [16] and a reply made by Chari [17], one of Gulati's collaborators. Efficient separation of morphine, M3G and M6G has been accomplished by an initial clean-up on a pre-column of underivatized silica followed by analysis on a C_{18} RP-HPLC column. The analysis time was less than 6 min [18]. A rapid simultaneous determination of morphine, M6G and M3G in serum using native fluorescence detection has been described [19]. A microassay which employs an 80 μl sample size has also been developed for the analysis of these three compounds in guinea pig plasma. Conventional RP-HPLC using acetonitrile–buffer (pH 2.1) (90:10) was preceded by clean-up using SPE C_{18} cartridges [20]. A rapid and highly automated determination of morphine, M6G and M3G in plasma or cerebrospinal fluid has been reported. Samples were extracted using on-line SPE followed by RP-HPLC with fluorimetric detection (see Fig. 1). The limit of detection, using 400 μl of a biological matrix was 0.85 and 3.4 μg ml^{-1}, respectively, for M3G and M6G [21].

Two groups [22,23] have used liquid chromatography–mass spectrometry (LC–MS) (electrospray) to assay morphine and its two glucuronides, one of them claiming an analysis time of 10 min [23]. Using LC–MS (ion-spray), the simultaneous determination in serum of heroin, morphine and M6G and M3G has been reported. Clean-up was achieved with C_2 SPE columns [24]. A German group has used LC–MS (atmospheric pressure chemical ionization) to assay M6G, M3G morphine and 6-monoacetylmorphine in human body fluids. Clean-up was achieved by SPE using C_{18} cartridges, and analysis of morphine, M3G and M6G was effected by RP-HPLC using formate buffer (pH 3.0)–acetonitrile (90:10). The limits of detection for the glucuronides were 1 ng ml^{-1}, and the method was claimed [25] to be ''selective, sensitive and robust''.

2.1.2.4. *Steroids*. To render bile alcohol glucuronides detectable by UV, conversion into their *p*-bromophenacyl esters prior to HPLC analysis has been reported [26]. HPLC–MS (electrospray) has been used to determine the glucuronides of seven steroids, including testosterone, androsterone and estrone [27]. The separation and characterisation of the glucuronides of vitamin D_3 and 25-hydroxyvitamin D_3 have been achieved using RP-HPLC and UV detection [28].

The same group [29] have characterized the monoglucuronides of vitamin D_2 and 25-hydroxy-

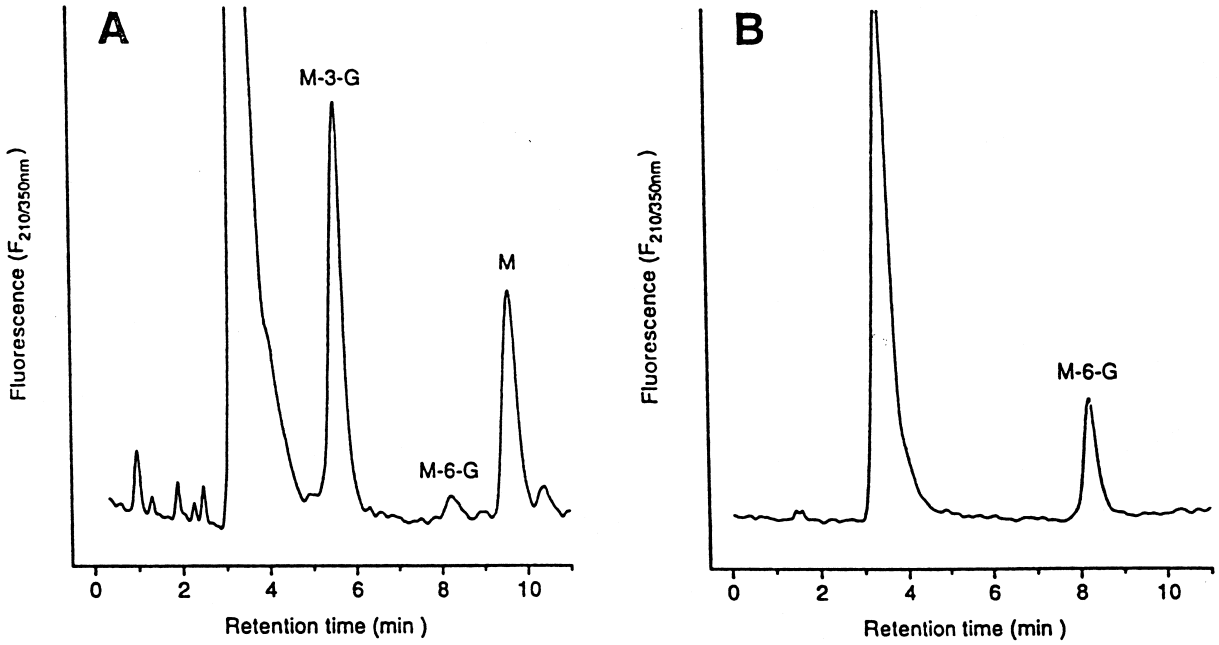


Fig. 1. (A) Representative chromatogram of a blood sample drawn 1 h after intravenous infusion of 10 mg morphine containing 12.8 ng ml^{-1} M-3-G, 3.4 ng ml^{-1} M-6-G and 8.3 ng ml^{-1} morphine. Sample size used for analysis was 400 μl. (B) Representative chromatogram of a blood sample drawn 1 h after intravenous infusion of 16 mg M-6-G containing 250 ng ml^{-1} M-6-G. Sample size was 100 μl. From Ref. [21] with permission.

vitamin D_2 in rat bile using HPLC–MS (chemical ionization), and the way is now clear to develop a quantitative HPLC assay.

A large, complex fluorimetric reagent which reacts selectively with carboxyl groups has been employed by Japanese workers [30,31] to determine some steroid glucuronides by RP-HPLC with fluorimetric detection. A di-imide is employed to effect the condensation reaction between the carboxyl group of the glucuronide and the acid hydrazide (RCO·NH·NH_2) group of the reagent. The fluorimetric grouping, R, is a substituted 2(1H)-quinoxalinone. In the most recent work [31], the assay of estriol 3- and 16-glucuronide was described. In principle, this is a general method for all glucuronides, but the reagent is somewhat esoteric – and perhaps expensive – and this may limit its wider use.

2.1.2.5. *Anti-cancer agents*. The glucuronide of 7-hydroxycoumarin has been determined in human plasma and serum by gradient elution RP-HPLC using a mobile phase consisting of aqueous acetic acid and methanol [32,33]. The method was subsequently used to monitor production of this glucuronide in liver homogenates [34] and has been developed by the same authors as a new procedure for the assay of β-glucuronidase activity in whole organ homogenates [35]. Comparative studies by this group showed that the analysis of this metabolite in urine is best conducted by CE (see Section 3.1.1).

The acyl glucuronides of three retinoids, all-*trans*-, 9-*cis*- and 13-*cis*-retinoic acid (RA) have been determined in a single-run analysis, which permits the simultaneous determination of the three parent RAs and a fourth congener, 9,13-*cis,cis*-RA [36]. Later work by another group led to the development of a method for the simultaneous assay of five glucuronides of RAs in rat urine, the three named above and those of 4-oxo-all-*trans*-RA and 4-oxo-13-*cis*-RA (see Fig. 2). The limit of detection was 0.01 μg ml^{-1} using 5 ml of rat urine [37].

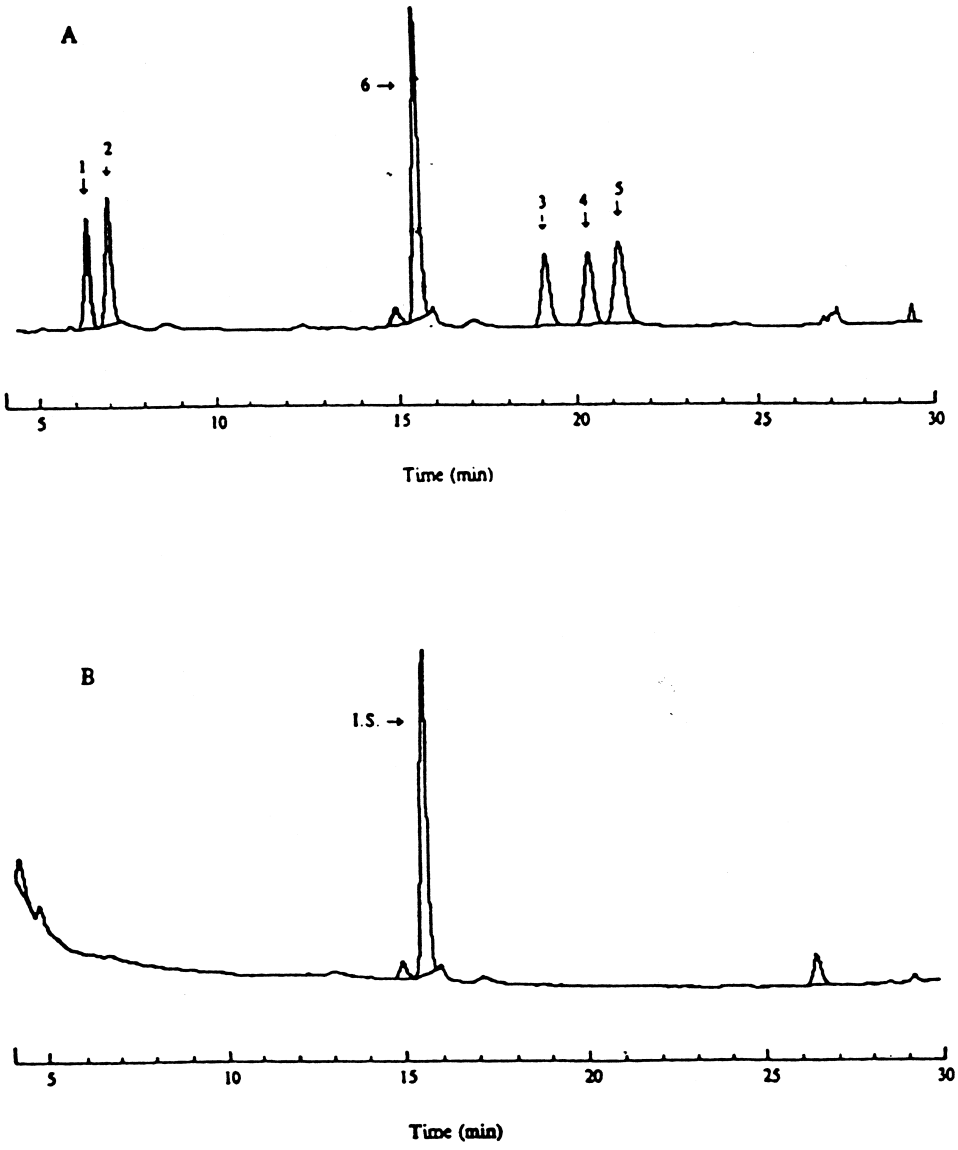


Fig. 2. (A) Chromatogram of a standard solution containing: 1=4-oxo-all-*trans*-, 2=4-oxo-13-*cis*-, 3=13-*cis*-, 4=9-*cis*-, 5=all-*trans*-retinoyl-β-glucuronides (104 ng each) and 6=internal standard (417 ng). (B) Chromatogram of drug-free rat urine fortified with 1000 ng of internal standard, acitretin-β-glucuronide. From Ref. [37] with permission.

2.1.2.6. *Anti-malarial agents*. β-Arteether, the ethyl ether of the natural anti-malarial agent, artemisinin, is being developed as an anti-malarial drug by the World Health Organization. Since the glucuronide conjugates of its metabolites may be biologically active, HPLC–MS has been employed to assay five that had become available by chemical and biochemical synthesis [38].

2.1.2.7. *Immunosuppressive agents*. Mycophenolate mofetil, the 2-(4-morphilino)ethyl ester of mycophenolic acid, is an immunosuppressive agent

used in organ transplant operations. In vivo, it is rapidly converted by esterases into mycophenolic acid (MPA), which is partly converted into its phenol glucuronide (MPAG). Assay of MPAG in human plasma by manual and automated RP-HPLC (see Fig. 3) was shown to have a limit of detection of 4.00 μg ml^{-1} [39]. Subsequently, in 1997, a robust method for the simultaneous determination of MPA and its glucuronide was described in which tetrabutylammonium was employed as an ion-pairing reagent. The limits of detection were 0.10 μg ml^{-1} for MPA and 0.80 μg ml^{-1} for its glucuronide [40].

2.1.2.8. *Miscellaneous drugs*. Azaperone, a tranquilliser in veterinary use, was extensively studied in rats. Three glucuronide metabolites were detected and identified by LC–MS–MS [41]. The β-blocking agent, oxprenolol, which is used therapeutically as the racemate, is converted, in rats and rabbits, into a pair of diastereoisomeric glucuronides. A simultaneous assay of each, in plasma or in liver homogenates, is attainable by RP-HPLC using a C_{18} column with UV detection [42].

Lamotrigine, a 1,2,4-triazine anticonvulsant derivative, forms a quaternary *N*-glucuronide in vivo. A successful assay was developed using sodium dodecyl sulphate (SDS) as an ion-pairing agent [43]. Two other quaternary *N*-glucuronides, those of amitryptyline and diphenhydramine, have been assayed using a C_{18} column [44]. The *N*-glucuronides of nicotine and of its metabolite, cotinine, have been separated and assayed [45].

Ion-pair RP-HPLC has been used to assay Zidovudine (3′-azido-3′-deoxythymidine; AZT) and

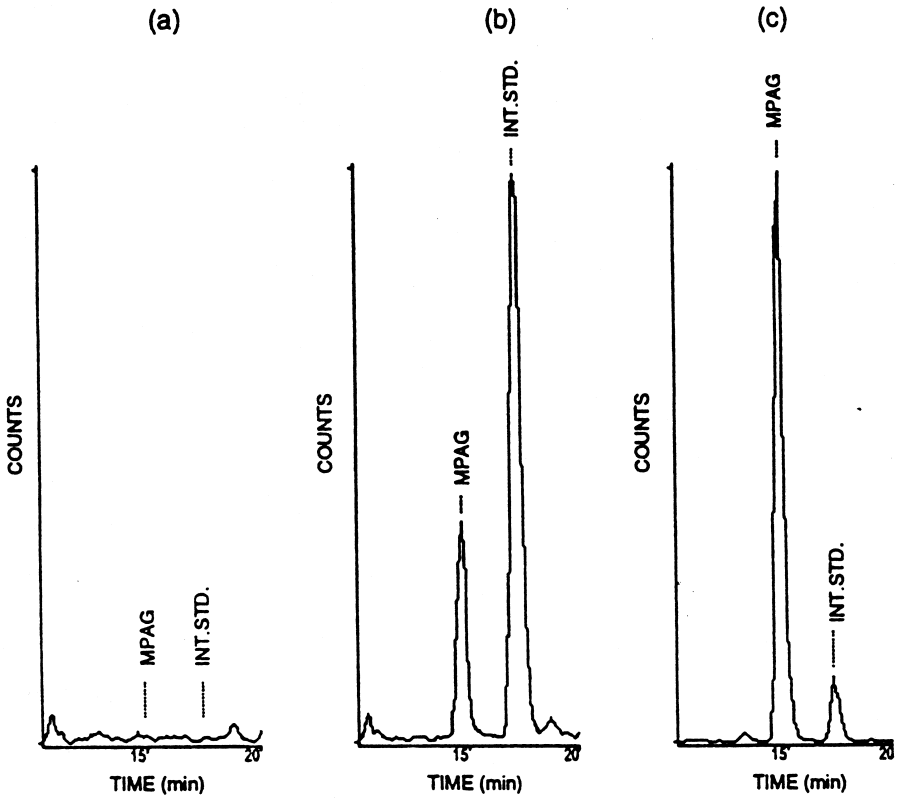


Fig. 3. Typical chromatograms obtained from the analysis of mycophenolic acid glucuronide (MPAG) in (a) blank human control plasma, (b) blank human control plasma spiked with 4 μg ml^{-1} of MPAG, or (c) plasma sample from a patient 1 h following oral administration of 1500 mg of mycophenolate mofetil twice daily. From Ref. [39] with permission.

its 5′-glucuronide. At pH 6.55, using 0.4% of octylamine in the mobile phase, the analysis was completed in 12 min [46]. Another group had already shown that the glucuronide of Zidovudine could be determined by RP-HPLC using a C_{18} column [47]. Furosemide, 4-chloro-*N*-(2-furylmethyl)-5-sulfamoylanthranilic acid, is converted into an acyl glucuronide in vivo. Assay of the glucuronide in human plasma and urine could be achieved by RP-HPLC using a C_{18} column and an acidic (pH 2.1) mobile phase. The limit of detection in plasma was 0.01 μg ml^{-1} [48]. The acyl glucuronide of furosemide has been subjected to a pH-rate profile determination. Greatest stability was seen at pH 3.0. Seven rearrangement products were identified [49].

The glucuronide of the anthracycline epidoxorubicin, and that of one of its metabolites epidoxorubicinol, may be determined in plasma by clean-up on C_2 SPE cartridges followed by RP-HPLC at pH 4.0 with fluorimetric detection [50]. The active metabolite of irinotecan, SN-38, can be converted into its glucuronide by human hepatic microsomes and the progress of the reaction can be monitored by RP-HPLC with fluorometric detection [51].

Directly-coupled HPLC-^{1}H nuclear magnetic resonance (NMR) in the ''stop flow'' mode has been used to separate and identify an equilibrated mixture of acyl-migrated glucuronide isomers of the model non-steroidal antiinflammatory agent, 6,11-dehydro-11-oxodibenz[*b*,*e*]oxepin-2-acetic acid in human urine. The HPLC conditions employed were gradient elution using 30–60% acetonitrile–deuterium oxide/phosphoric acid (pH 2.5) [52,53].

Thyroxine (T4) is converted into an acyl and a phenyl glucuronide by rat liver microsomes. Simultaneous assay of all three compounds is achievable by ion-pair RP-HPLC using a mobile phase at pH 1.8 [54].

2.1.3. Miscellaneous chemicals

2.1.3.1. *Miscellaneous O-containing compounds*. Free glucuronic acid, as a component of polysaccharide hydrolysates, may be assayed by HPAEC, using pulsed amperometric detection [55]. The glucuronides of 1- and 3-hydroxybenz[*a*]pyrene have been assayed in cell culture media using microbore HPLC–MS [fast-atom bombardment (FAB)] [56].

The glucuronides of phenol, *p*-nitrophenol and β-naphthol have been determined in the perfusate from isolated perfused rat preparations using ion-pair RP-HPLC [57]. An assay for 4- methylumbelliferone and its glucuronide using a C_8 column has been developed as a probe for human glucuronidase activity in tissue homogenates [58]. An isocratic RP-HPLC system has been developed for the assay of phenolphthalein and its glucuronide in plasma [59]. A second group has reported a RP-HPLC method for assaying the same compounds in the serum, bile and plasma of dogs [60].

2.1.3.2. *Miscellaneous N-containing compounds*. Although conventional ion-exchange HPLC and ion-pair RP-HPLC using tetrabutylammonium hydroxide allowed ready determination of uridine 5′-diphospho-α-D-glucose and its hydrolysis products, neither method was suitable for the determination of uridine 5′-diphospho-α-D-glucuronic acid (α-UDPGA). However, mixed ion-pair RP-HPLC, using a combination of tetrabutylammonium–tetraethylammonium hydroxide (1:1), has been found to offer a convenient and effective means (see Fig. 4) of simultaneously determining α-UDPGA and its hydrolysis products, UDP, UMP and cUMP [61].

The glucuronides of 2-aminophenol, 4-nitrophenol and phenol have been separated from their parent phenols by coupled-column micellar RP-HPLC. The surfactant added to the mobile phase to achieve micellar conditions was cetylmethylammonium bromide [62].

2.1.3.3. *Miscellaneous halogen-containing compounds*. The separation of the mixtures of positional isomers and anomers of 2-, 3- and 4-fluorobenzoic acid glucuronides has been achieved using an ODS-2 column and a mobile phase of phosphate buffer (pH 7.4) containing 1% acetonitrile [63]. The neutral buffer was superior to an acidic buffer for optimal separation of the positional isomers, but on-column mutarotation of injected samples could not be avoided.

In an important application of this work, a joint Danish and British group has employed directly-coupled HPLC-^{1}H NMR in the ''stop flow'' mode to

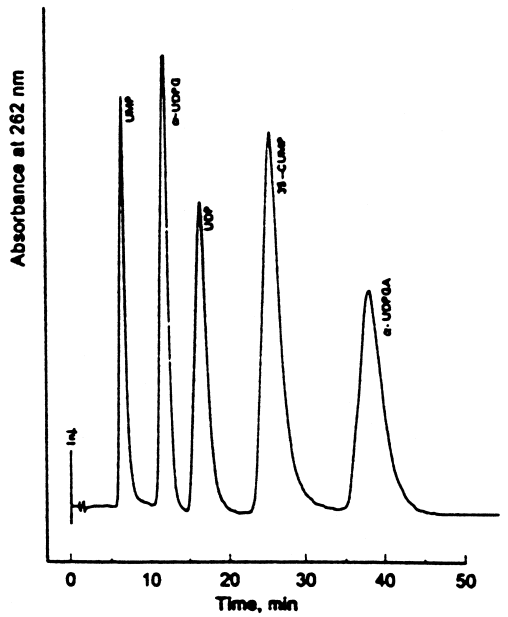


Fig. 4. Mixed ion-pair reversed-phase HPLC trace of a mixture of UMP, α-UDPG, UDP, cUMP and α-UDPGA. Conditions: 10 μl from an aqueous solution of about 1 mg ml^{-1} injected by valve onto a 200×4.5 mm column packed with Partisil 10 ODS 2. The mobile phase was 0.002375 *M* tetrabutylammonium hydroxide, 0.002375 *M* tetraethylammonium hydroxide, 0.0475 *M* ammonium dihydrogenorthophosphate, adjusted to pH 6 with KOH in 10% aqueous methanol. From Ref. [61] with permission.

separate and identify an equilibrated mixture of ester glucuronide isomers of 2-fluorobenzoic acid. The HPLC conditions were developed to provide optimal compatibility with the NMR assay. For example, water was largely replaced by deuterium oxide in the mobile phase. The conditions finally chosen were an isocratic system composed of acetonitrile–0.2 *M* phosphate buffer (pH 7.4)–deuterium oxide (1:10:89, v/v/v) with a flow-rate of 1 ml min^{-1} [64].

The method was subsequently used [65] to determine the rate constants of the isomerisation reactions. ^{19}F NMR can also be used in HPLC–NMR ''stopped flow'' mode – as well as in ''continuous-flow'' mode. Such studies have used a mixture of the ester glucuronides of 2- and of 3-trifluoromethylbenzoic acids as model compounds to demonstrate the rapidity with which the isomers can be identified and have charted the progress of the acyl migration reactions of individual isomers. ^{19}F NMR data, collected over a period of 14 h, of the 4-*O*-(3-trifluoromethylbenzoyl)-D-glucuronate incubated at pH 7.4 in phosphate buffer, for example, showed that the first-formed products were the α and β anomers of the 3-*O*-acyl isomers, with subsequent formation of the α and β anomers of the 2-*O*-acyl isomers (see Fig. 5) [66].

2.2. Gas chromatography

A new GC–MS procedure for the assay of glucuronic and iduronic acids in glycosaminoglycans involves reduction of their carboxyl groups with $NaBD_4$ followed by silylation [67]. The 3-glucuronides (and the 3-glucosides) of several bile acids have been assayed by capillary GC, after derivitization by methylation and silylation [68].

The glucuronide of ethanol persists in man longer than ethanol, and a method has therefore been developed for its analysis in human urine. An internal standard of 5-D-ethyl glucuronide was used in the GC–MS method that was developed [69]. A minor metabolite of estrone, estrone-3-glucuronide has been assayed in serum and urine by a GC–MS method involving acetylation; the carboxyl group, strangely, was left underivatized [70].

2.3. Electrochromatography

This is the description given to a novel type of chromatography, which takes its cue from electrophoresis in the sense that a large voltage is deployed to facilitate separation. As a result of a series of pilot experiments, the method is claimed to offer considerable potential for the analysis of aromatic glucuronides [71].

2.4. Countercurrent chromatography (CCC)

''Reversed-phase'' CCC (butanol–bicarbonate buffer, pH 8.64) has been used to separate and isolate the sulfate and glucuronide conjugates of *p*-nitrophenol [72].

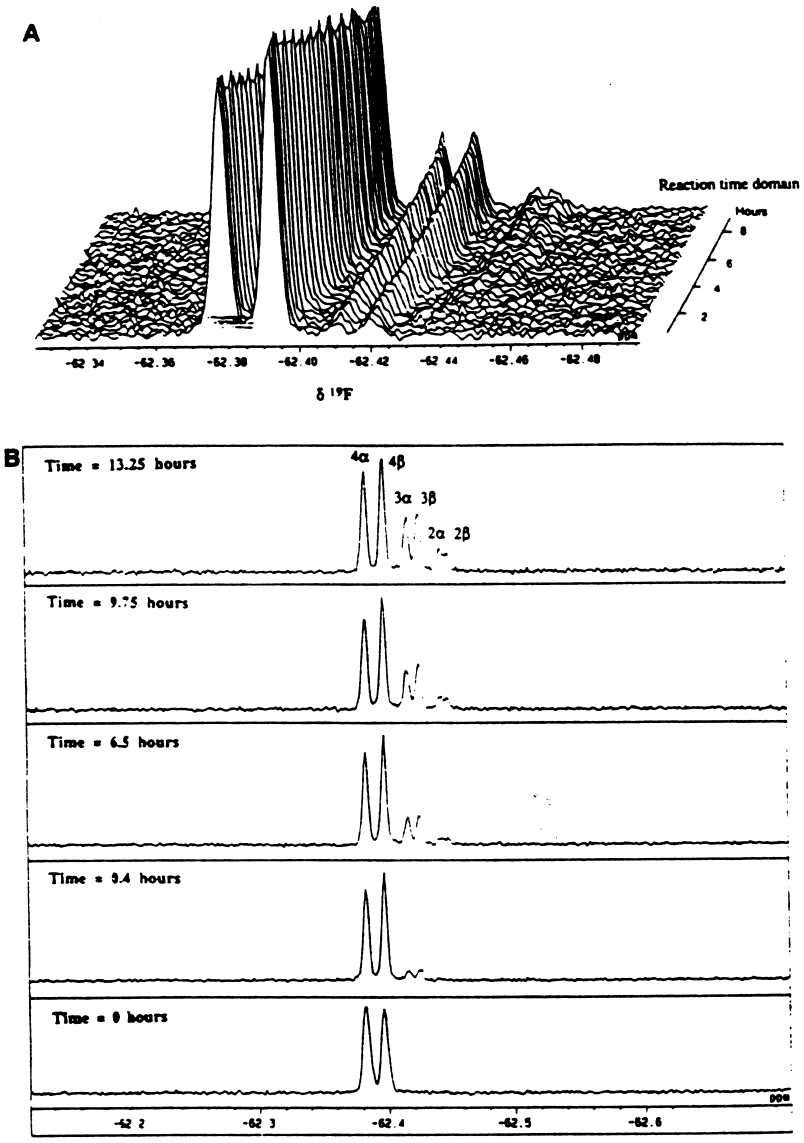


Fig. 5. (A) The stacked plot of a ''dynamic'' stop-flow HPLC-^{19}F NMR run, with NMR chemical shift on the horizontal axis and the reaction time-course on the orthogonal axis. The acyl migration reactions of the 4-*O*-acyl-3-trifluoromethylbenzoic acid acid isomer were followed at pH 7.4 (292 K). (B) Individual spectra picked at different time points of the reaction time. The assignments of the peaks are indicated on the top spectrum α-4-*O*-acyl isomer (4α), β-4-*O*-acyl isomer (4β), α-3-*O*-acyl isomer (3α), β-3-*O*-acyl isomer (3β), α-2-*O*-acyl isomer (2α) and β-2-*O*-acyl isomer (2β). From Ref. [65] with permission.

3. Electrophoretic methods

3.1. Drugs

3.1.1. Opioids

Direct determination of morphine-3-glucuronide in human urine by capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) has been achieved with a limit of detection of 20 μg ml^{-1} [73]. A systematic study of the separation behaviour of free and glucuronidated opioids by CE in aqueous, binary and micellar media has been reported [74]. The 12 opioids studied, including three glucuronides, could not be fully

separated by aqueous or binary CZE. However, they could be resolved completely by aqueous EKC at pH 10.6 [74].

Using M3G as a model compound, Taylor et al. [75] have developed a MECC method to monitor directly glucuronide hydrolysis by commercial glucuronidases. The same group have reported a MECC method for the assay of M3G in equine urine samples. Using a clean-up by SPE, the limit of detection was 150 ng ml^{-1}. Chromatograms obtained by MECC analysis of post-administration and spiked urine showed that M3G was easily identified among all the background peaks (see Fig. 6) [76].

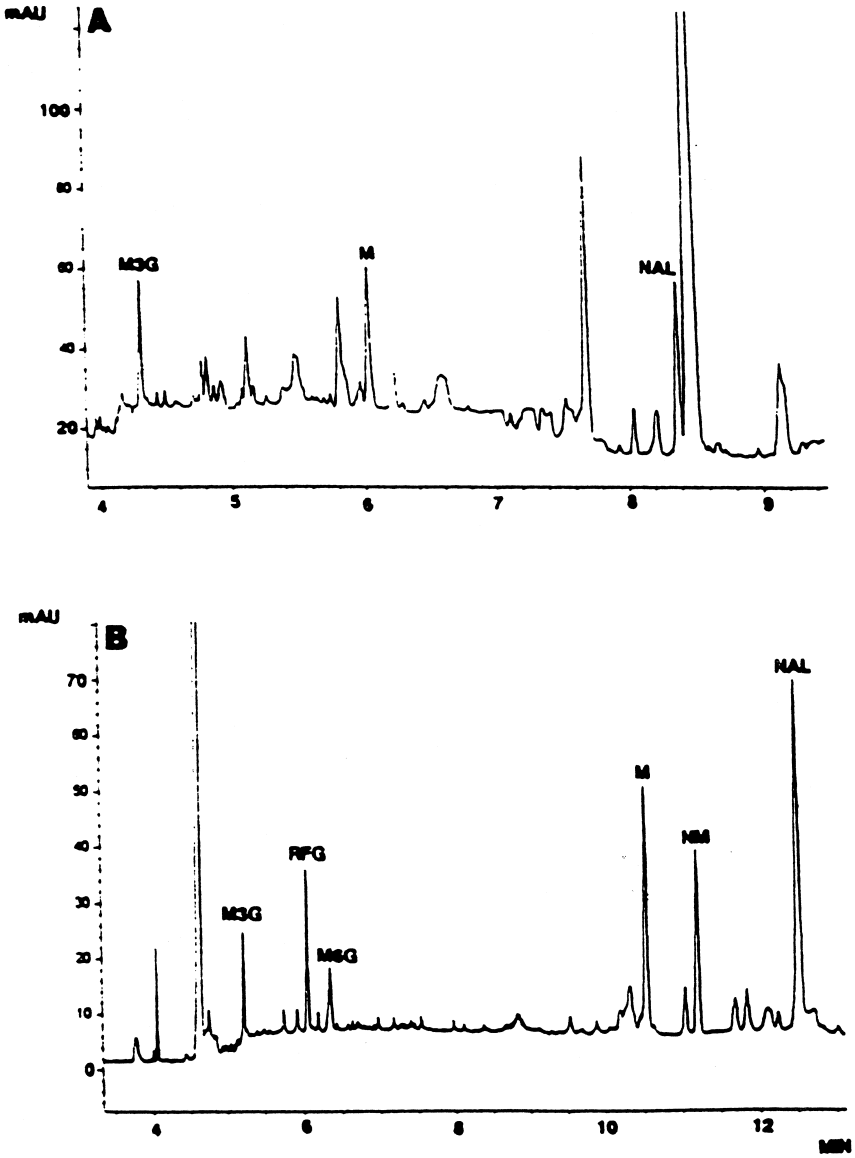


Fig. 6. Chromatograms from MECC analysis of equine urine samples extracted by SPE. A=Post-administration urine, B=spiked urine (1 μg ml^{-1}). MECC performed at 25°C in HP fused-silica capillary (48.5 cm×50 μm I.D., 150 μm I.D. detection window light path). Buffer=Borate (70 m*M*, pH 9.3); SDS (70 m*M*). Injection by pressure (150 mbar s^{-1}). Field=470 V cm^{-1}. Detection by UV (195 nm). In run A, acetonitrile (5%, v/v) was added to run buffer. From Ref. [75] with permission.

3.1.2. Anti-cancer agents

A direct CE method, without sample clean-up, has been developed for the simultaneous determination of 7-hydroxycoumarin and its glucuronide in human urine. Separation was achieved on an uncoated silica capillary at 20 kV using 0.1 *M* phosphate buffer (pH 7.0)–acetonitrile (90:10) containing sodium deoxycholate. The only pretreatment of urine samples was low-speed centrifugation for 5 min, and the limit of detection of the glucuronide was 5 $\mu g\ ml^{-1}$ [77].

3.1.3. Miscellaneous drugs

The glucuronide conjugate of wogonin, a flavanoid component of several Chinese herbal drugs, may be assayed by CE [78].

4. Conclusions

The most commonly used HPLC stationary phase for the assay of glucuronide conjugates is C_{18}, with C_8 finding only minor use. The most popular mobile phase is composed of acetonitrile and an acidic buffer, usually at pH 1.8–4.0. This ensures that the carboxyl group of the glucuronide moiety is fully protonated when it encounters the stationary phase, a generally accepted pre-condition for good chromatography of acids. Felicitously, the use of an acidic mobile phase also minimises the acyl-migration processes that the 1-β-acyl glucuronides can undergo – which are known to occur readily under neutral or alkaline conditions and to be minimal at pH 3.0 [49]. Generally, isocratic elution conditions are suitable, although gradient elution is often required if a simultaneous determination of a glucuronide and a non-polar parent compound is needed. Occasionally, ion-pairing agents such as tetrabutylammonium are added to the mobile phase to improve resolution [11,40,46,54,57,61]. There is a single example of the use of coupled-column micellar RP-HPLC, which was achieved by adding cetylmethylammonium bromide to the mobile phase [62].

The detector component of the HPLC system is usually UV–spectrophotometric, but occasionally fluorometric. For compounds which lack a chromophore, such as steroids, derivatisation of the glucuronide via formation of its *p*-phenacyl ester is useful [26]. A method of attaching a fluorophore to the carboxyl group of the glucuronide moiety has also been reported [30,31]. The highly selective detectors, MS and NMR spectroscopy, are also being used, with impressive effect. The power of LC–MS is well known, and several examples of its use confirm its unrivalled combination of selectivity and sensitivity [22–25,27–29]. HPLC–NMR, by contrast, is relatively new and largely untested. However, the practical utility of HPLC–NMR in the characterization of drug glucuronide isomers in whole biofluids has been clearly demonstrated. The identification of the α and β anomers of the 2-, 3- and 4-acyl glucuronides of fluoro- and trifluoromethylbenzoic acid has been achieved using ''stop-flow'' methods [64,66], and has pointed the way to studies of those drug glucuronides that are known to elicit toxic effects via covalent interaction of their acyl-migration products with important biomolecules.

The problem of clean-up of biofluid samples for HPLC assay is an important one. Although it is not a component of this review, the ''minuscule chromatography'' that SPE represents is a recurring feature of many of the articles reviewed. Indeed, the use of C_{18} SPE cartridges for clean-up is widespread – and they have become virtually indispensable when rapid and semi-automated analyses are required.

CE is becoming important, not least because the clean-up of body fluid samples is simple or can be dispensed with. However, since it cannot match HPLC in sensitivity it remains a minor technique for the assay of glucuronide conjugates.

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