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Analytical considerations for trace determinations of drugs in breast milk

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Abstract

Recent scientific and regulatory interest in lacteal excretion of drugs has prompted this review of bioanalytical sample preparation techniques for milk. The composition and properties of milk are reviewed, with emphasis on how the sample preparation is affected. The most important principals of mammary gland pharmacology, including protein binding, ion trapping and liquid solubility, are described. Because adequate milk volume is difficult to obtain from some smaller rodent species, special arrangements for sample collection, control preparation and assay standardization often need to be made. Several commonly-used sample preparation approaches for drugs in milk, including direct injection, dialysis and ultrafiltration, protein precipitation, liquid–liquid extraction, solid-phase extraction and immunoaffinity extraction, have been reported with varying degrees of success. The advantages and disadvantages of each of these approaches is discussed.

Keywords: Dialysis; Direct injection; Drug disposition; Liquid chromatography; Liquid–liquid extraction; Matrix; Milk; Protein precipitation; Sample preparation; Solid-phase extraction

1. Introduction

From both regulatory and scientific perspectives, it is becoming increasingly more important to characterize the excretion of pharmaceutical substances into breast milk. A recent report issued by the *International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use* [1] states that "Areas where more basic research would be useful for optimization of test designs are male fertility assessments, and kinetics and metabolism in pregnant/lactating animals." This perspective is strengthened by fundamental scientific questions that arise during drug development. Such questions include: To what extent is a neonate exposed to a drug during lactation? Is it best to begin dosing during or after gestation, and during or after breast feeding? These questions are addressed to some degree through nonclinical studies involving lactating animal models such as

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rabbit, rat or mouse. Although it is risky to extrapolate from animal models to human therapy, the animal studies can offer guidance in study design, dosing regimen construction, and to what extent a therapeutic drug could be excreted in milk. To support studies of drug excretion into milk, it is important that quantitative bioanalytical methodology be crafted to fit this unique matrix. This requires a knowledge of the properties of milk and the factors that affect lacteal drug excretion.

Milk has high but variable protein, fat and carbohydrate contents. Its composition depends not only on the mammalian species from which it is acquired, but also on diet, the number of days since the onset of lactation and on other factors. These variables can affect the recovery of an extracted drug, and also the method precision, accuracy and sample compatibility with a subsequent chromatographic technique. Numerous factors affecting the rate and extent of lacteal drug excretion have been identified [2,3]. Both passive diffusion and active secretory mechanisms of excretion have been reported [4]. These variables will affect the level of drug in the milk, and ultimately, the sensitivity requirements of the bioanalytical method.

A number of sample cleanup techniques, such as ultrafiltration, protein precipitation, liquid–liquid extraction, solid-phase extraction (SPE) and immunoaffinity extraction, have been reported for use with milk prior to liquid chromatographic separation. The selection of a sample preparation technique depends on its ability to handle a complex and variable matrix, compatibility with subsequent chromatography, and on the selectivity and sensitivity requirements of the assay. Human breast milk is relatively easy to obtain, but milk from small species such as monkey, rat or mouse must be considered as precious and requires special attention for standardization and control preparation.

This paper considers each of the areas described above, and will, hopefully, give the bioanalytical chemist sufficient background to begin construction of a robust quantitative method for determining drugs in milk from humans and other species.

2. Composition and properties of milk

Milk is a specialized extracellular suspension produced by female mammalians after gestation. It is intended as a primary source of nourishment for the neonate. Milk is unique in that it is a nutritionally complete natural food containing proteins, lipids, carbohydrates, vitamins and minerals. Although all mammals produce milk of similar composition, the relative abundance of certain milk components varies considerably with the time of day, the day of lactation and, for pharmacological studies, the dosing regimen and pharmacological properties of the drug [3–8]. For example, the protein content of normal rat milk can vary from 8% to 13% (w/w) over a 28 day period, and the fat content can vary from 6 to 20% [5]. The fluctuations of protein and fat in rat milk as functions of lactation day are shown graphically in Fig. 1. A study of the changes in composition of human milk over the term of lactation has also been reported [6].

A summary of the average milk composition for seven selected mammalian species is given in Table 1. The largest single component of all mammalian milk is water, which comprises between 65% and 90% of the weight for the seven species shown [9,10]. The remaining components of milk are dissolved or suspended solids consisting of proteins, lipids and carbohydrates.

The major milk protein, casein, is present at the low percent level. Along with emulsified lipids, the calcium salt of casein is responsible for the white appearance of milk. Bovine β-casein is a medium-sized protein containing 209 amino acid residues, with a molecular weight of 23 600 Da. Cystein and cystine residues, and therefore disulfide bridges, are conspicuously absent from the molecule. Casein is readily hydrolyzed into its constituent amino acids, making it a ready dietary source for these nutrients [11].

With an isoelectric point of 4.7, casein can be precipitated from milk by addition of lactic acid, a process similar to that used for making cheese. The remaining supernatant, or whey, contains numerous proteins which are more hydrophilic and more difficult to precipitate. These include α-lactalbumin, lactoferrin, immunoglobulins, lac-

Table 1
Average milk compositions of selected mammalian species [9,10]

Constituent	Percent composition[a]						
	Human	Cow	Monkey	Dog	Mouse	Rat	Rabbit
Water	87.6	87.3	84.6	76.5	70.7	69.1	67.2
Solids	12.4	12.7	15.4	23.5	29.3	30.9	32.8
Protein	1.0	3.4	1.6	7.9	9.0	11.8	13.9
Casein	0.4	2.8	1.1	5.8	7.0	8.5	[b]
Lipids	3.8	3.7	4.0	12.9	13.1	14.8	18.3
Carbohydrates	7.0	4.8	7.0	3.1	3.0	2.8	2.1
Ash	0.2	0.7	<0.1	1.2	1.3	1.5	1.8
pH	7.0	6.7	[b]	[b]	[b]	6.6	[b]

[a] g per 100 g or g per 100 ml with no correction for specific gravity.
[b] Data not available.

toperoxidase, xanthine oxidase, lipase, proteases, alkaline phosphatase, and polypeptide growth factors [12,13]. Some of the more prominent non-protein components of whey include carotenes, vitamins A, C, and D, riboflavin, thiamine, niacin and pantothenic acid. Lactose, uniquely present in milk, accounts for most (95–98%) of the carbohydrates. The lipids are predominantly triglycerides, dispersed as small globules. Many saturated fats are present, as are phosphoglycerides and cholesterol, in trace amounts [11].

Colostrum, a milk-like fluid produced during the first few days of lactation, is significantly different from milk in that it contains 2–4 times more protein, mostly in the form of immunoglobulins. Colostrum can be yellow or orange, due to the presence of β-carotene, a vitamin A precursor. Carotenes are 50–100 times more abundant in colostrum than in milk. Greater amounts of riboflavin, niacinamide and other vitamin precursors are also present in colostrum [11].

In summary, milk is a complex biological matrix that contains nearly as many different components as plasma. It can be more difficult to handle than plasma due to high, variable lipid and protein contents. The high lipid content can be troublesome for some extraction and chromatographic techniques. The highly variable or transitory composition of milk can make assay reproducibility difficult to achieve. The challenge is to develop a sample cleanup procedure that can handle the high protein and lipid contents and is insensitive to the variable composition.

3. Factors affecting drug concentrations in milk

The lacteal excretion of most therapeutic drugs is governed by simple, passive diffusion. Only a few drugs, such as cimetidine and benzylpenicillin, are known to undergo active transport [4,14–16]. No less than 18 factors that affect the rate and extent of drug excretion into breast milk and subsequent consumption by the neonate have been identified. These factors have been described by Wilson [2] and by Pons et al.[3]. Briefly, these factors fall into five general areas: (1) the pharmacology of the drug in the mother; (2) the physiology of the breast; (3) the composition of the milk; (4) the nutritional demands and pharmacology of the neonate; and (5) the physical–chemical properties of the drug in the matrix. Three of the most important biopharmaceutical factors that influence lacteal drug excretion—protein binding, ion trapping and lipid solubility—are briefly reviewed here.

3.1. Protein binding

Although active transport is possible for a few drugs, most blood plasma components reach milk by passive diffusion across alveolar cells in the

breast. Very small (<200 Da) molecules can also pass through aqueous pores between cells to reach the milk. Because protein-bound plasma components are too large to pass through the cellular membranes, only the free drug fraction is available for transfer across this barrier. Some protein binding occurs in milk, but generally the extent of protein binding is only 20%–60% of that for plasma [17,18].

3.2. Ion trapping

Milk is slightly more acidic than maternal blood, and so consequently weak bases ionize to a greater extent in milk than in plasma. A neutral molecule that passes from plasma to milk and becomes ionized will be unable to return to the systemic pool; hence it will be trapped in the milk. By this mechanism, weakly basic drugs can concentrate in milk.

3.3. Lipid solubility

Fat-soluble drugs will dissolve preferentially in the lipid component of milk and, therefore, may not be available for diffusion back into plasma. Because of this higher preference for the lipid component of milk, and because milk is the only fat reservoir that is periodically emptied, milk can be a very effective sink for fat-soluble drugs.

4. Sampling and assay considerations

Fig. 2 shows the relative milk yield for various mammalian species versus body weight [2]. Although there is some individual and between-species variability, a proportionality between daily milk yield and body weight does exist. Larger species of pharmacological importance, such as rabbit, monkey, dog and human, can produce several hundred milliliters of milk per day. Collection of adequate sample volume and control matrix is relatively easy to achieve. It is much more difficult, however, to collect adequate volumes of sample and or blank matrix from rat and, especially, mouse. An adult, female Sprague–Dawley rat, a preferred strain for milk collection, can

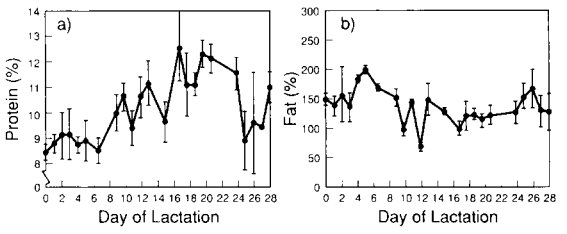

Fig. 1. Developmental patterns of (a) milk protein and (b) milk fat concentrations in the rat. Each point represents 3–14 animals (mean ± standard error), except for days 26–28, which represents 1–2 animals (from Ref. [5]).

produce 2–5 ml of milk over a 15–30 min period, and a total of 15–25 ml per day. This amount could be collected from each of several rats to produce an adequate control matrix pool. Lactating female rats can be purchased from commercial suppliers for $60–75 each. Several procedures for obtaining milk from a lactating rat have been published. A typical procedure has been described by Keen et al. [5].

Mice, however, produce only about 3 ml of milk per day. If drug disposition into mouse needed to be studied, it would be necessary to pool control milk from many mice or to use the sample as its own control, as in standard addition approaches [19]. Micro-analytical approaches such as in-vivo-online microdialysis–capillary electrophoresis [20] could be useful, provided

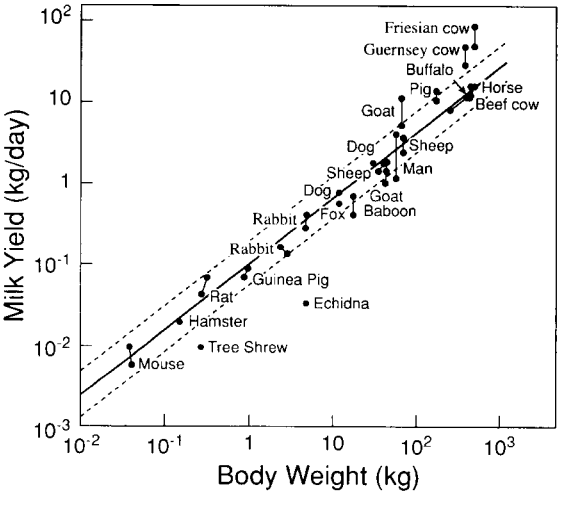

Fig. 2. Milk yields for different species (from Ref. [2]).

there is adequate sensitivity for detection. Low drug-to-protein binding is required for this approach to work. Another approach would be to substitute milk from an alternate species for standardization and/or controls. Rat milk is a likely substitute for mouse milk because of the similar composition (Table 1); as with mouse milk, rat milk has a high solids content.

If possible, the researchers should verify that assay recovery, precision, accuracy and specificity are consistent under extremes of milk composition. If they are not, then additional method development may be required. Better buffering of samples and more effective disruption of fat globules are two areas which merit attention. As with plasma assays, milk from several animals (at various stages of lactation) should be tested for adequate method specificity. Approaches for dilution of concentrated samples (parallelism studies) should be tested and verified for precision and accuracy, especially if the fat composition varies as samples are diluted.

Once milk samples are obtained, difficulties in their handling centers around the need to release drug and metabolites from the milk fat. If this release is inadequate, then extraction efficiency and precision may be poor. A secondary task is disruption of protein binding and removal of protein from samples. This is generally less difficult than for plasma samples because protein binding in milk is much less than in plasma. With the same extraction procedure, chromatograms from milk are often cleaner than those from plasma, serum or urine.

5. Commonly used sample cleanup techniques for milk

A number of modern sample cleanup techniques have been applied to the determination of xenobiotics in milk. The majority of this literature deals with veterinary drugs and toxins in bovine milk, but human therapeutic agents in nonhuman and human milk are also represented. The most commonly described sample cleanup approaches include: direct injection (with or without column switching) [21,22]; ultrafiltration or dialysis [23–26]; protein precipitation [27–33]; liquid–liquid extraction [14,34–50]; solid-phase extraction [51–58]; and immunoaffinity extraction [59–61]. Representative examples of these approaches are discussed below.

5.1. Direct injection

Until the advent of the internal surface reversed-phase (ISRP) sorbent, direct injection of milk into a liquid chromatograph had only very limited success. Dadgar and Power [21] describe the necessity of centrifuging samples prior to chromatographic injection, to separate the lipid (upper) layer from the aqueous (middle) and protein (bottom) layers. This approach was possible only because the analytes, antihistamines containing ionized amines, partitioned poorly into the lipid layer. A portion of the aqueous layer was siphoned from the tube and directly injected. The extra work involved in the centrifugation step made the omission of a deliberate protein precipitation, a step which would add considerable robustness, questionable.

The use of an ISRP column to effect a true direct injection of milk (50 μl) into a reversed-phase system [22] has also been described. This chromatographic sorbent consists of porous polymer beads with a selected hydrophobic phase bound to the internal pore surfaces. Retention is achieved by analyte penetration through the pores of the sorbent to the inner surface where partitioning can take place. Matrix molecules, such as large lipids and proteins, which are too high to reach the inner surfaces are poorly retained and elute at or near the column void. As is often true with non-silica-based supports, some reduction in column efficiency is sustained, relative to silica-based sorbents. Unfortunately, ISRP columns are useful only under limited ranges of pH and organic modifier concentrations.

5.2. Ultrafiltration and dialysis

Ultrafiltration and dialysis are simple and rapid, although they are not highly selective sample cleanup techniques. They rely on the passage of low molecular weight drugs, that are not extensively protein-bound, through a membrane. De-

pending on the molecular weight cutoff of the selected membrane, high molecular weight components do not pass. Using ultrafiltration, Ohkubo et al. [23] obtained high (91%–100%) recoveries for the antihypertensive drug propylthiouracil down to 100 ng ml^{-1} in human milk. A column-switching routine was used to improve the selectivity of the separation. Better detection limits are not normally attained with ultrafiltration or dialysis unless combined with additional sample cleanup procedures, or a better means of detection. Quantitation limits for this technique are often limited by chemical noise.

5.3. Protein precipitation

Protein precipitation has been used frequently for milk sample cleanup prior to HPLC, because the approach is simple and generally effective. One shortcoming of this technique is that it does not sufficiently remove the lipids, which are present in large concentrations in milk from most species. As a result, protein precipitation cleanups are most effective when combined with a step that will remove these lipids. A hexane wash or a SPE are two effective approaches, which are discussed in Sections 5.4 and 5.5 respectively.

An illustration of protein precipitation was reported by Kearns et al. [33], who described a procedure for determining the novel cephalosporin, cefpirome, in human milk. The sample pretreatment portion of this assay involved protein precipitation by addition of a 4:1 ratio of isopropanol to milk. The sample was vortexed and centrifuged. The supernatant was evaporated to dryness and resuspended in mobile phase prior to injection into a reverse-phase LC system. As is often the situation with protein precipitation procedures, only modest quantitation limits (625 ng ml^{-1}) were obtained. Other method performance features included high recovery ($\geq$94%) and acceptable precision and accuracy (<2.8% RSD and bias) over the calibration range. In principle it would be difficult to obtain long-term reliability with this method, because no consideration is given to removal of the milk lipids prior to LC injection.

5.4. Liquid–liquid extraction

Liquid–liquid extraction is the most widely used sample cleanup technique for determining drugs and metabolites in milk. With varying success, it has been applied to the extraction of analgesics [35,37], β-adrenergic blockers [36,39, 41], androgens [38], xanthines [40,42,44,47], tricyclic antidepressants [43], benzodiazepines [45,46], anticonvulsants [50] and other xenobiotics [48,49]. Because of the wide range of immiscible organic solvents available, liquid–liquid extraction can be tailored to specific analytes, thereby improving assay selectivity.

Due to the high fat content of milk relative to other biological fluids, an aqueous back extraction of the organic phase is often necessary to rid the extract of lipids. If lipids remain after evaporation to dryness and resuspension with a reversed-phase compatible solvent, they could phase-separate in the sample. If injected, these lipids globules would partition strongly into the stationary phase and would need to be periodically flushed out with an organic solvent to prevent deterioration of the chromatography column. Alternatively, it is possible to remove lipids from the milk with a hexane wash before analyte extraction, provided that the analyte is ionized or otherwise has low extractability into hexane, as described by Dostal et al. [14] or Greene et al. [62].

A representative liquid–liquid extraction/back extraction procedure has been reported by Stebler and Guentert [46], who used diethyl ether in a 15:1 volume ratio to extract benzodiazepines from buffered (0.067 M phosphate, pH 7.4) human milk. The ether phase was separated, acidified with 1.5 M HCl, and decanted from the acidic aqueous phase. The aqueous phase was basified with 2 M NaOH and extracted with additional diethyl ether. This ether phase was evaporated to dryness, and the residue resuspended in mobile phase. Recoveries of 84%–92% were attained for diazepam, nordazepam and flurazepam from human milk. Quantitation limits were 20 ng ml^{-1}, with inter- and intra-day inaccuracy and imprecision of better than 8%, using UV detection at 241 nm. Chromatograms from this work are shown in

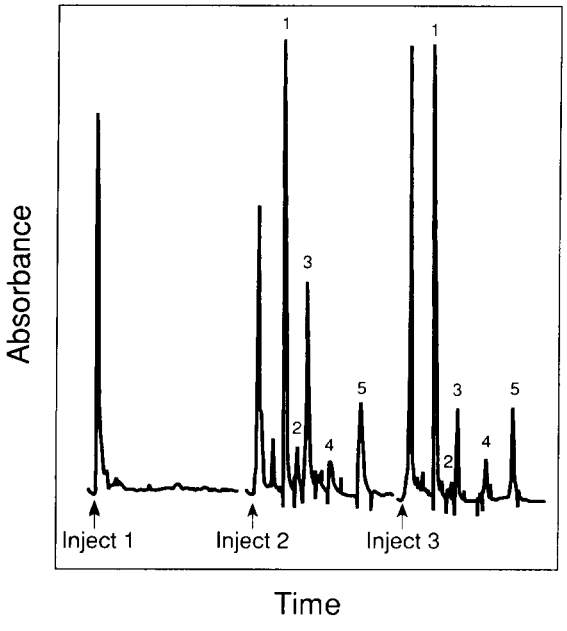

Fig. 3. Chromatograms of liquid–liquid extractions of blank human milk ("Inject 1"), standard mixture of benzodiazepines in human milk (100 ng ml^{-1} in each component ("Inject 2")), and milk from a NZW rabbit 24 h after a 2 mg kg^{-1} diazepam dose, followed by 1.4 mg h^{-1} over 26 h ("Inject 3"). Peaks (1) fluorazepam (retention time 3.0 min); (2) oxazepam (3.8 min); (3) nordazepam (4.5 min); (4) temazepam (6.3 min); (5) diazepam (8.6 min) (from Ref. [46]).

Fig. 3 for three milk extracts: (1) blank human milk; (2) a standard mixture of benzodiazepines in human milk; and (3) milk from a NZW rabbit after a diazepam dose. Chromatographic separation was achieved by a reversed-phase LC system.

Liquid–liquid extraction is an effective sample cleanup for milk, but prior hexane washing or labor-intensive back-extractions are generally required to obtain adequate method ruggedness.

5.5. SPE

SPE has been applied to milk sample cleanup with or without prior protein separation steps. Those papers that incorporate a protein separation step prior to SPE do so by lowering the sample pH with dilute hydrochloric or perchloric acid. The resulting suspension is centrifuged, and the supernatant is transferred to a reversed-phase SPE cartridge in the usual manner. Levamisole [56] and phenprocoumon [57] have been extracted in this way.

Several papers by the Japanese team of Ohkubo et al. [54–56] involve acidification (hence protein precipitation) of milk prior to loading the suspension onto a reversed-phase SPE cartridge. In this way, the bulk of the milk proteins are denatured and removed by the cartridge. If the analytes are then eluted with as weak a solvent as possible (typically 60% methanol–water), most milk lipids will be retained by the sorbent. With this approach, these workers have been able to routinely quantify concentrations as low as 5 ng ml^{-1} of several psychoactive drugs with high ($\geq$92%) recovery. Representative HPLC–UV (254 nm)

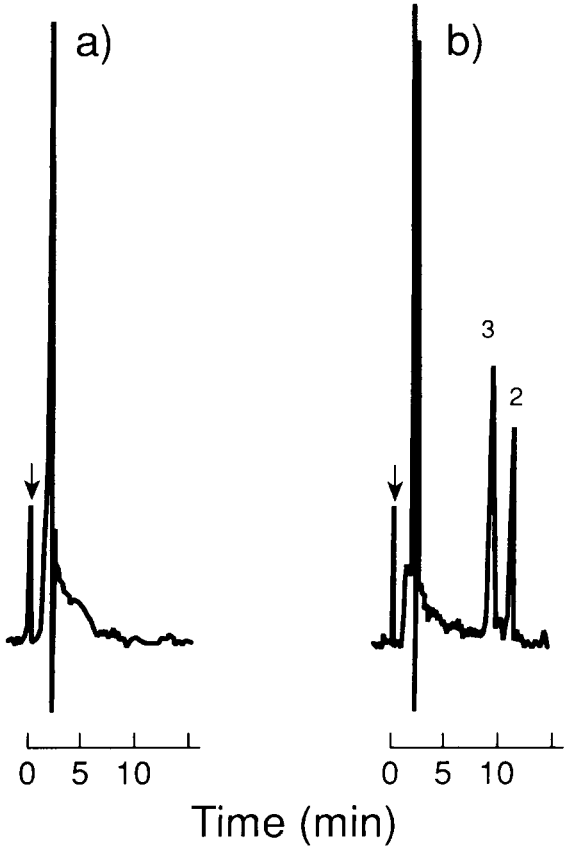

Fig. 4. Chromatograms of SPE of (a) blank human milk and (b) human milk spiked with (3) internal standard and (2) haloperidol (50 ng ml^{-1}) (from Ref. [54]).

chromatograms of these extracts are given in Fig. 4. These workers also demonstrated that it is possible to perform SPE of haloperidol in human breast milk with only buffering and dilution before SPE loading [54]. This approach appears to be convenient and effective (5 ng ml^{-1} quantitation limit, <9% inter-assay RSD).

Extraction of lipophilic compounds from milk can result in poor recovery unless the fat globules are destroyed prior to extraction. One way to achieve this is by addition of a water-miscible solvent to the milk. An investigation of this approach was recently reported by Laganan et al. [58], who added methanol to milk (1:1 v/v) and then sonicated to disrupt the globules. Samples were further diluted with water to a final methanol concentration of 5% before loading on graphitized carbon SPE cartridges. Recoveries of eight triazine herbicides ranged from 73%–92%, with RSDs of 3%–5%. Although this approach has not been reported for use with therapeutic drugs, it could prove useful in the future.

SPE offers the opportunity to remove both proteins and lipids from the milk sample without the need for prior organic washes or protein precipitation. It should continue to be widely applied for cleanup of milk samples.

5.6. *Immunoaffinity extractions*

At least three papers describing immunoaffinity extraction and application to milk samples have been described. In one representative paper [59], chloramphenacol was extracted from porcine milk using an online immunoaffinity column, coupled to a C8 analytical column with a switching valve network. The milk was preprocessed by skimming off the fat layer, then precipitating the proteins with 15% trichloroacetic acid and separating the proteins from the supernatant. The supernatant was basified, filtered and injected (5 ml). The immunoaffinity column contained monoclonal antibodies raised against chloramphenicol. Under normal phosphate–isotonic saline conditions, the immunoaffinity column retained the analytes and allowed matrix components to flush to waste. A step gradient that consists of glycine–sodium chloride was used to elute the analytes onto the analytical column, followed by a reversed-phase separation and UV detection (280 nm). Less than 1 $\mu g\ kg^{-1}$ of chloramphenicol in milk could be quantified with a mean recovery of $70\% \pm 6\%$. Although a fairly elaborate setup is often required, immunoaffinity chromatography can offer spectacular cleanup for milk and other complex biological samples, with good analyte recovery. It has been underutilized for trace determinations of drugs.

6. Conclusions

Continuing basic research on lacteal drug excretion is required to answer fundamental scientific questions regarding neonate exposure. To this end, a cogent sampling and sample cleanup approach is often needed for milk. For models employing smaller animals, milk samples can be volume-limited, thus requiring one of the less common calibration and control techniques. To minimize errors associated with variable milk composition, assay precision, accuracy, specificity and recovery should be verified over the composition range.

Although milk is a complex and variable matrix, containing large amounts of protein and fat, successful LC assays have been developed using standard sample cleanup approaches. Solid-phase and liquid–liquid extraction continue to be used effectively for sample cleanup and trace enrichment, providing the analytes are released from the lipid fraction. To protect the LC column, removal of the lipid fraction from the extract is strongly recommended. Other sample cleanup techniques, such as direct injection, ultrafiltration, dialysis or protein precipitation, are less effective at removing lipids and are, therefore, used less often. Although elaborate, immunoaffinity chromatography offers an elegant approach to milk cleanup and could be used more extensively in the future.

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