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Influence of methodological aspects on tissue drug concentration estimation

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Abstract

Fundamental studies in tissue drug extraction have been made using rokitamycin as a test probe. Radiolabelled rokitamycin was administered intravenously to 12 rats. Lungs and femurs were excised a few minutes later. Rokitamycin was extracted from lungs using six procedures (based either on ion-pairing, dissolution or deproteinization) whose performances (mean recovery and reproducibility) were compared. Three grinding procedures were also compared for the extraction of rokitamycin from bone: pulverization by a magnetic stirring bar in a liquid nitrogen bath, slicing into small pieces and crushing with pestle and mortar. The effect of binding proteins (albumin or α_1-acid glycoprotein) in the extraction mixture was also evaluated. Magnetic stirring bar grinding was the most efficient. Deproteinization was necessary to obtain the highest recovery, but the agent had to be chosen carefully. Binding proteins either had no effect or decreased the recovery of rokitamycin. Recovery from bone was lower than that from lung. Binding to cellular components in the post-extraction pellet was only 3% (lung) and 9% (bone). It is concluded that a careful optimization of the extraction procedure of a drug from a tissue allows quantitative and reproducible measurement of its concentration.

Keywords: Antibiotics; Methodology; Tissular concentration

1. Introduction

Antibiotic tissue penetration is often measured to compare agents. Many authors [1–5] have described the pitfalls of quantifying antibiotic distribution by use of tissue homogenates. Recently, Nix et al. [6] discussed the relevance of antibiotic tissue penetration and its impact on infection response. Tissue concentrations might be useful to compare antibiotics when bacteria are intracellular, or extracellular but in a site which has a permeability barrier and does not obey the laws of passive diffusion [7]. The usual method for assessing tissue penetration involves homogenation of whole tissue and measurement of the antibiotic concentration in the supernatant. However, analytical recovery of the drug in the supernatant may be incomplete because of drug binding to cell components or drug loss during handling. To overcome this problem, determination of the analytical recovery of the drug by adding a known amount of the drug to a representative tissue sample and performing the assay is often advocated. However, drug binding to cellular components could be different depending on whether the drug is added in vitro to tissue homogenates or penetrates the tissue in vivo [8]: the temperature, pH, protein content and concentration of the complexing cations are

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different. This difference in incorporation is even more problematical for drugs that penetrate cells, e.g. clindamycin, macrolides, rifampin and fluoroquinolones [9,10]. Validation of the analytical procedure is thus difficult. One way to measure the analytical recovery is to use the radiolabelled drug.

In this study, a ^{14}C-labelled macrolide structurally related to josamycin, rokitamycin, was administered to rats, extracted from lungs (representative of a soft tissue) and bones (representative of a hard tissue) by different procedures, and the analytical recovery measured. Rokitamycin was chosen as a test because macrolides are well-known to penetrate the cells, a factor likely to hinder complete extraction. In rats and humans, rokitamycin is transformed in LMA7 by hydrolysis of an acetyl group, and further metabolized in LMV by hydrolysis of a propionyl group. The rationale for testing the different procedures of rokitamycin extraction is based on the physicochemical properties and pharmacokinetics of this macrolide [11]. Rokitamycin is a basic drug which is highly soluble in usual non-aqueous solvents other than hexane. Its pK_a value is 7.5 and its solubility in water decreases from 250 mg ml^{-1} at pH 1.0 to 0.08 mg ml^{-1} at pH 7.0. Its octanol/water distribution coefficient also depends on pH and ranges from 15 (pH 3.0) to 1200 (pH 7.0). The corresponding values for rokitamycin metabolites, LMA7 and LMV, have not been measured, but owing to their chemical structures, their pK_a values are expected to be similar to that of rokitamycin, while their solubilities in water are expected to be higher and their distribution coefficients lower. Binding of rokitamycin, LMA7 and LMV to human plasma is, respectively, 85, 40 and 15% [12]. The performances (mean yield and reproducibility) of the different homogenation and extraction procedures were compared, giving a new insight into some of the factors influencing the determination of antibiotic tissue concentrations.

2. Materials and methods

2.1. Animals and samples

12 male Row-Wiston rats (Iffa-Credo, L-Arbresle, France) were used the day after their arrival. Their mean ± SD weight was 213 ± 6.3 g (range 205–223 g). They were anesthetized with 0.16 ml of a 5% pentobarbitone solution injected intraperitoneally. The ethanolic solution of ^{14}C-labelled rokitamycin 100 μCi ml^{-1} (Toyo Joso, Japan) was diluted with an equal volume of saline solution and each rat received 0.4 ml in a hind leg vein. A few minutes later (mean ± SD lag time 13.7 ± 2.7 min; range 8–16 min), the animals were sacrificed by cardiac puncture and exsanguination. Femurs and lungs were excised. Lungs were divided into three equal portions to provide 36 pieces which were cleansed of surface blood with gauze and then weighed. The mean ± SD weight of the lung pieces was 568 ± 56 mg (range 453–697 mg). The samples were then pulverized with a magnetic stirring bar in liquid nitrogen bath using a Spex grinder (Spex 6700, Bioblock, Strasbourg, France).

Femurs were also divided into three equal portions to give 36 pieces which weighed 726–197 mg (range 243–1186 mg).

2.2. Lung extraction procedures

Six extraction procedures were assayed. Each procedure was applied to six lung pieces from different rats. In all cases, about 200 mg of accurately weighed lung tissue powder was shaken for 15 min with one of the extraction solvents described in Table 1, and centrifuged for 10 min at 1000 g. In procedures 1 and 2, 0.1 ml of the supernatant was used to measure the radioactivity of extracted rokitamycin. In procedures 3–6, the supernatant was collected, evaporated to dryness and the residue dissolved

Table 1
Composition of the extraction solvent used in each procedure

Procedure	Extraction solvent
1	1.5 ml of ammonium acetate (10 mM; pH 4.5) +0.1 ml saturated NaCl +0.1 ml of sodium laurylsulphate (10 mM)
2	1 ml of phosphate buffer (67 mM; pH 6.5) standing for 3 h at +4 °C
3	3 ml of methanol
4	3 ml of acetonitrile– phosphate buffer (67 mM; pH 6.5) (20:80, v/v)
5	3 ml of ammonium acetate (20 mM; pH 4.5) +3 ml of acetonitrile–methanol (2:1, v/v)
6	10 ml of phosphate buffer (20 mM; pH 7.0) +3 ml of acetonitrile–methanol (2:1, v/v)

in 0.5 ml of an acetonitrile–phosphate buffer (67 mM; pH 6.5) (20:80, v/v) mixture, of which 0.1 ml was used to count the radioactivity. Unextracted rokitamycin was measured by measuring the radioactivity in 0.2 ml of the post-extraction pellet.

2.3. Bone-grinding procedures

Three procedures were assayed, and each was applied to six femur pieces from different rats. In procedure A, bones were pulverized in a liquid nitrogen bath using the Spex 6700 grinder. In procedure B, the bone specimens were sliced into small pieces as described by Leigh [13]. In procedure C, the bones were crushed using a pestle and mortar with an equivalent volume of screened sand. After grinding, rokitamycin was extracted from the pulverized bones using a solvent described for procedure 6 and counted.

2.4. Influence of proteins on bone extraction

The influence of albumin or α_1-acid glycoprotein (AGP) on rokitamycin extraction from bone was assessed as follows. Three groups of six femur pieces were ground according to procedure A described above. In all cases, about 400 mg of bone powder was shaken for 15 min with 2.5 ml of a 20 mM pH 7.0 phosphate buffer containing either no protein (procedure A1), 500 mg l^{-1} human AGP (Sigma, ref. G9885, l'Isle d'Abeau, France) (procedure A2) or 40 g l^{-1} human albumin (sigma, ref. A1653) (procedure A3). 2.5 ml of an acetonitrile–methanol (2:1, v/v) mixture was then added. After 15 min of shaking, the tubes were centrifuged for 10 min at 1000 *g*. The supernatant from each tube was collected, evaporated to dryness and the residue dissolved in 0.5 ml of an acetonitrile–phosphate buffer (67 mM; pH 6.5) (20:80, v/v) mixture. The radioactivity was measured as described above.

2.5. Calculation of the extraction yield

The rokitamycin extraction yield, R, was determined according to the formula:

$$R = \frac{D_e S_e}{D_e S_e + D_t S_t}$$

where S_e and S_t are, respectively, the signals (desintegration per min) emitted by the extraction mixture and the tissue; D_e and D_t are factors correcting for the dilution in the scintillation liquid of the extraction mixture and tissue, respectively. No correction was made for the amount of macrolide in the blood remaining in the tissues, because it accounted for less than 2% of the extracted rokitamycin (data not shown).

2.6. Adsorption onto sand

Drug adsorption onto the sand used in procedure C was evaluated, taking as examples two non-steroidal antiinflammatory drugs of the propionic acid family, namely minalfen and ketoprofen. One-millilitre aliquots of human plasma were spiked with an aqueous solution of minalfen to yield two six-point standard curves, namely 0, 2, 5, 10, 20 and 40 mg l^{-1}. In one set of standard, 1 g of sand was added to each standard, and minalfen was determined by high pressure liquid chromatography (HPLC) as described by Paillet et al. [14]. The same procedure was applied to ketoprofen. The slope of each calibration curve was calculated by linear regression.

2.7. Statistical analysis

Mean extraction yields obtained using each procedure was compared by one-way analysis of variance (ANOVA). Two-by-two comparisons between the means were made using the Scheffe F test. The level of significance was 0.05.

3. Results

Rokitamycin extraction yields form lung tissue obtained by the six procedures are shown in Table 2. Three groups can be distinguished: procedures 4, 5 and 6 in which the extraction is nearly complete and highly reproducible (the relative standard deviation, RSD, is in the order of 1–2%); procedures 1 and 2, in which the extraction is high (about 80%) and the reproducibility is still good (RSD = 1–6%); and procedure 3, in which the extraction is poor and not reproducible (RSD = 91%). ANOVA revealed a significant difference between the mean yields ($P = 0.0001$); Scheffe's test showed that the yield of procedure 3 was significantly lower compared to all the other procedures. Rokitamycin extraction yields from bone with the three grinding procedures are shown in

Table 2
Yield (R) of rokitamycin extracted from lung or bone according to the procedure used

Procedure	Mean R	SD	Range
Lung			
1	0.82	0.01	0.81–0.83
2	0.77	0.05	0.67–0.80
3	0.43	0.39	0.01–0.95
4	0.95	0.03	0.88–0.97
5	0.96	0.004	0.95–0.96
6	0.97	0.01	0.94–0.98
Bone			
A (magnetic) [a]	0.91	0.04	0.86–0.97
B (slices) [a]	0.84	0.02	0.80–0.86
C (sand) [a]	0.87	0.02	0.84–0.89
A1 (no protein) [b]	0.88	0.02	0.85–0.90
A2 (AGP) [b]	0.89	0.02	0.86–0.92
A3 (albumin) [b]	0.83	0.03	0.79–0.86

[a] According to the grinding procedure.
[b] According to the protein content of the extraction solvent.

Table 2. The overall difference between the three groups was significant ($P = 0.0025$). Procedures A and C were different, but not significantly; the yield from procedure B was significantly lower than those of A and C. Although the three procedures are highly reproducible (RSD = 3%), the magnetic stirring bar grinding in liquid nitrogen afforded the highest yield, although the difference with pestle crushing in sand was not significant. The extraction yield from bone was significantly lower than that from lung ($P = 0.001$), as shown by the comparison of procedures 6 and A (Table 2). Procedure A was then slightly modified so that the effect of proteins in the extraction solvent on the extraction yield could be evaluated. The overall difference between the three grinding techniques was significant ($P = 0.0007$). AGP, which is one of the macrolide-binding proteins, did not significantly improve the rokitamycin extraction yield from bone compared to phosphate buffer, whereas albumin unexpectedly decreased the extraction yield significantly compared to the two other procdures (A3 vs. A1 and A2).

Minalfen and ketoprofen were significantly absorbed onto the sand used in procedure C: slopes of their calibration curves were, respectively, 1.61 times ($P = 0.005$) and 2.41 times ($P = 0.001$) lower than those observed without sand.

4. Discussion

Since rokitamycin and its metabolites retain the radioactive carbon nucleus, they were measured en bloc by the radioassay and only an overall extraction yield could be determined. However, a radioassay was used because this is the only way to measure conveniently the absolute extraction yield of a drug at low concentration. Moreover, since LMA7 and LMV are active metabolites, their extraction from tissues is also of interest. The degradation of rokitamycin and its metabolites during extraction appeared unlikely. Indeed, it was shown earlier that these antibiotics could be measured in tissues by a specific HPLC assay after extraction by procedure 1 [15], and the results were in good agreement with those obtained by microbioassay after extraction by procedure 2.

4.1. Rokitamycin extraction from lung

No precipitation of proteins occurred in procedures 1 and 2. In procedure 1, rokitamycin and its metabolites were solubilized by ion-pairing with laurylsulphate at pH 4.5, as described previously [15], while in procedure 2, rokitamycin and its metabolites are expected to dissolve in water because 90% of them are in the protonated form at pH 6.5. Despite these facts, the extraction yield was only about 80% in both cases, which could be explained by adsorption onto or binding to cellular components. Therefore, in procedure 3, methanol was used to ensure denaturation and precipitation of proteins, but the resultant extraction yield was low and highly variable; this could be more or less explained by trapping of rokitamycin in the precipitate. Finally, use of various combinations of a deprotinizing solvent with an aqueous buffer (procedures 4–6) gave very high yields with good reproducibility, regardless of the pH of the buffer, i.e. irreversible binding of the macrolide to cellular components occurred, if at all, to a very minor extent.

4.2. Rokitamycin extraction from bone

Application of the same grinding and extraction procedure to lung and bones gave a slightly lower yield with the latter ($P = 0.001$). Thus, the structure and composition of tissues can influence the extraction yield of xenobiotics. Extraction procedures have to be validated in each case.

Magnetic stirring bar-grinding, which gave the finest powder, also gave the highest extraction yield; conversely, slicing, which resulted in larger pieces, was the least efficient method. The size of the powder granules seems to be a critical factor in the extraction yield from bone. One additional argument in favour of magnetic stirring bar grinding with Spex 6700 is that it proceeds at liquid nitrogen temperature (−196°C), and thus might prevent thermal degradation of drugs (according to the manufacturer, the temperature of the sample increases by 15 °C min^{-1} during the procedure). Nevertheless, this factor had no influence on our results since the radioassay measured the parent drug and its metabolites. Thermal degradation could be relevant to drugs like cefotaxime or josamycin, which are known to be unstable in biological samples at ambient temperature. In these cases, grinding in a liquid nitrogen bath could result in much higher extraction yields than other procedures.

Pestle and mortar crushing using sand as an adjuvant afforded good yields. However, adsorption onto sand is always a potential pitfall; sand contains silica, whose adsorption properties are well-known. To illustrate this point, adsorption of two drugs from the same family was studied. Although minalfen and ketoprofen are highly bound to plasma proteins (more than 99%) [16], they were so extensively adsorbed onto sand that their respective apparent concentrations in plasma were divided by a factor of 1.41 and 2.61. Although minalfen and ketoprofen are structural analogues, their adsorption onto sand was very different. Therefore, when drug concentrations in tissue are measured in a chromatographic assay, internal standardization should be used with caution. Adsorption of the drug and its internal standard onto the tissue and other components of the extraction system should be investigated. Use of in-vitro spiked tissues to calibrate the assay could be an elegant procedure to overcome these difficulties, but the tissue used in vitro for calibration should be the same as that from which the drug is extracted (i.e. same organ and species). However, drug binding to cellular components after in vitro spiking may be different from that occuring after administration of the drug to a living animal.

Another way to obtain a high recovery might be to incorporate some proteins into the extraction mixture of the drug. If these proteins have a high affinity for the drug, the latter should be displaced from its reversible binding to cellular structures. One drawback of this approach is that the proteins have to be removed before drug measurement, without loss of drug. However, when using plasma proteins such as albumin or AGP, the binding is almost always reversible and the analyst is only faced with a problem very similar to the usual one, i.e. extracting a drug from plasma. Because macrolides are known to bind to AGP (specific and saturable binding) and albumin (non-specific and non-saturable binding) [17], these proteins were used at their physiological concentration to explore their influence on rokitamycin extraction yield. Compared to the extraction solvent containing no protein, AGP provided no improvement, while albumin lowered the extraction yield. This unexpected result could be explained by the precipitation of the extraction protein after the extraction step. Again, rokitamycin and/or its metabolites could be adsorbed onto or trapped in the albumin defecate, resulting in a lower final extraction yield.

Another way to promote a high extraction yield of a drug from a tissue is to use protease enzymes to hydrolyze proteins, such as cathespines. However, use of enzymes involves a period of incubation at mild temperature and may hydrolyze some amide bonds of the drug, two factors that risk the promotion of degradation of the drug. In particular, some families of antibiotics incorporate amino acids in their structure, e.g. penicillins, cephalosporins, glycopeptides, polymyxines, while others are thermally unstable (cefotaxime, josamycine). Therefore, this approach could be of value, but only in some cases, and was not evaluated here.

Finally, this work has clearly demonstrated that an intracellular drug like rokitamycin can be almost quantitatively extracted from a soft tissue (lung) or a hard tissue (bone), provided that the extraction conditions have been optimized in each case. Conversely, inappropriate conditions (e.g. procedure 3) can result in a low and highly variable extraction yield (0.43 ± 0.39). Although the results of this study cannot be extrapolated to other drugs because of differences in pK_a, lipophilicity, partition coefficients, protein-binding, etc., it demonstrates that to achieve complete extraction, the method of tissue homogenation, the modification of the matrix sample and the extraction procedure per se have to be optimized in order to prevent degradation and adsorption of the drug onto cellular components.

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